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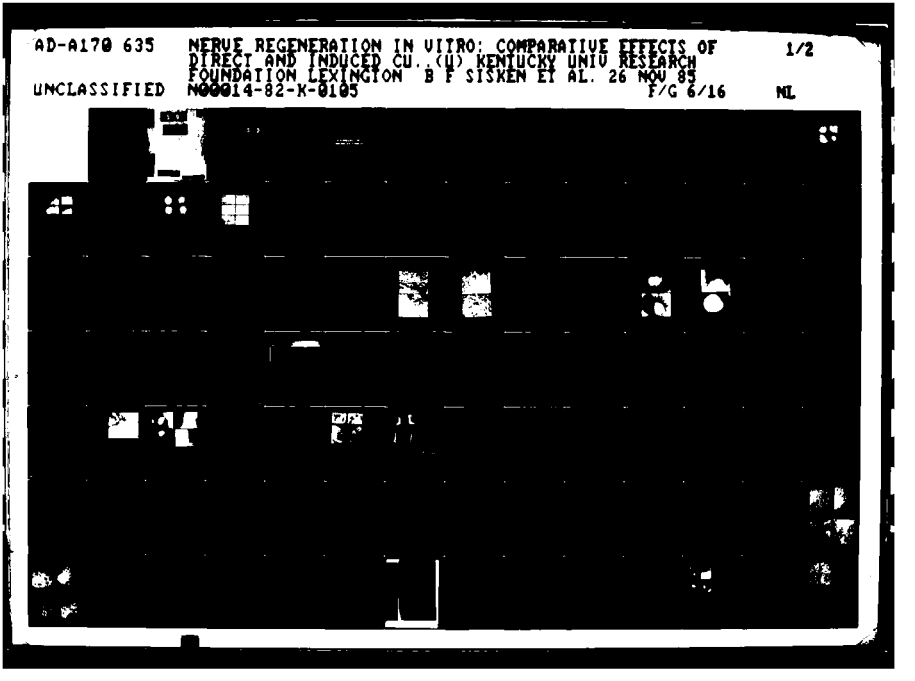

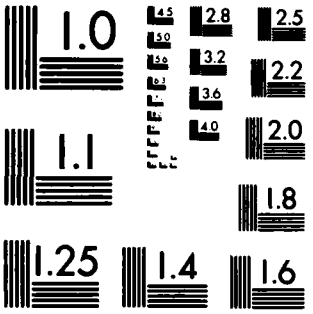

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19. ABSTRACT (Continue on reverse if necessary and identify by block number)

Our investigations have focused on the response of sensory neurons (DRG) obtained from young (8 day) and older(14 day) chick embryos and 15 day old rat fetuses to low levels of direct (DC) or induced (PEMF) current or NGF. Direct current (10 nA) was applied continuously for 3 days through tantalum electrodes immersed in the media; current was induced by exposing cultures to pulsed electromagnetic fields (PEMF) using a single pulse, 72 Hz, 12 hrs/day for 2 days with coils supplied by Electrobiology Inc. The data presented in the Appendix indicates the following: in 8 day cultures, the growth response obtained with NGF, PEMF or DC was significantly increased over controls at 3 days and this was maintained only with NGF or DC by 6 days. PEMF effects were correlated with current density only when the coils were oriented vertically. Significantly-increased protein content and incorporation of ^{3}H-proline/ ganglia paralleled the increased growth response obtained with DC. In older (14 day) DRG, neurite outgrowth was enhanced only with DC. The growth response in 15 day fetal DRG was not influenced by PEMF or DC; only NGF stimulated growth.

Neurophysiological studies found the resting potential of the DRG neurons to range from 30-60 mV; they exhibited overshooting potentials upon intracellular stimulation. Equivocal results were found in membrane potential changes when DC was applied.

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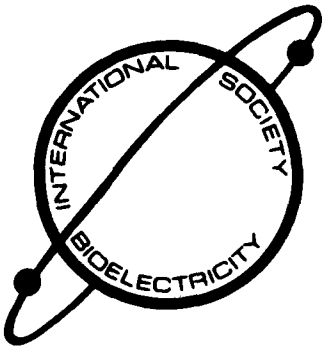
19. ABSTRACT (Continuation)

Conductivity of tissue culture media was determined and constant current devices were fabricated to generate a growth response curve as a function of current density. Constant current levels of 30-60 nA (30-60 nA/cm^2) produced the maximal growth response. To determine if DC-stimulation of growth is correlated with ion changes, ion substitution/ionophore studies were performed. Inhibitors of calcium influx (lanthanum, Verapramil) mimicked the DC-growth stimulation; increased external calcium or ionophore A23187 which allow calcium entry inhibited neurite outgrowth. These results agree with many reports of the deleterious effects of high levels of intraneuronal calcium and support our hypothesis that one mode of action of non-depolarizing levels of DC is to prevent calcium entry and indirectly promote growth and increase in protein content.

Other areas of study partially supported by this contract and found in the Appendix include: 1)DC-effects on neuroblastoma (N18) cells, 2) DC and PEMF stimulation of central nervous system (spinal cord) regeneration in culture, 3) PEMF effects on sciatic nerve regeneration in the rat, and (4)DC plus neural tissue implant stimulation of bone growth in amputated rat limbs.



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EXPERIMENTAL NEUROLOGY 88, 27–43 (1985)

Triethanolamine, Tris, Hepes, and Cytosine Arabinoside Show Neuritogenic Activity in Cultured Chick Embryo Ganglia

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Neuritogenesis, which occurs to a slight extent in chick embryo ganglia maintained under standard conditions and which is maximally stimulated by nerve growth factor, also was enhanced by presence in the medium of buffers (triethanolamine, Tris, and Hepes) and cytosine arabinoside and by the passage of direct electric current. The major effect of the buffers probably was to remove protons from cell membranes, that of the current to produce accelerated movement of ions through membranes of the ganglionic cells, and that of cytosine arabinoside to decrease the numbers of nonneural cells by inhibiting DNA synthesis. The buffers were neuritogenically ineffective on nerve growth factor-sensitive PC12 pheochromocytoma cells in culture. Media from ganglia in which triethanolamine or passage of electric current had elicited outgrowth of neurites produced no observable effect on PC12 cells under our experimental conditions. Current data fit the hypothesis that, whereas nerve growth factor exerts direct neuritogenic effects on neurons, the other treatments affect neural–nonneural interactions, possibly by way of gap junctions or changes in direct physical contact, so as to disinhibit inherent neural neuritogenic potential and/or to stimulate it.

INTRODUCTION

The original isolation of nerve growth factor (NGF) was greatly facilitated by the development of an assay system with cultured chick embryo ganglia

Abbreviations: NGF—nerve growth factor, TREA—triethanolamine, ara C—cytosine arabinoside, DRG—dorsal root ganglion.

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utilizing as endpoint the semiquantitative estimation of the degree of extension of a halo of neurites from the ganglia in response to extracts containing NGF (21). Under the usual conditions of culture, there is only little fiber extension from ganglia obtained from 8-day embryos. The ganglia consist of mixtures of neuronal and nonneuronal cells which, under *in vivo* conditions, may mutually affect each other in many ways by exchanging a variety of chemical substances or by exerting physical forces on each other, for example, pressure, adhesion, repulsion, and others. The receptivity to NGF of some of the neuronal elements in the cultured ganglia is indicated by remarkable responses to very small amounts of this substance added to the culture medium. The known competence of glial cells in the ganglia (23, 25, 38, 39) and possibly other cell types (9, 14, 15) to produce and release NGF and/or other neuronotrophic substances is not strongly expressed under "classical" *in vitro* culture conditions.

From an already voluminous and still rapidly expanding literature it is apparent that NGF, which produces observable effects on membranes of receptive cells as early as 30 s after initial contact (8), exerts pleiotypic effects typical of those expected when a rate-limiting or primary reaction (a bottle-neck so to speak) is released that consequently enables the occurrence of cascades of reactions to take place that have reverberations throughout the cell, from cell membrane to nucleus. The release is triggered by the interaction of NGF with specific receptors on cell membranes (12), which probably is a *sine qua non* for its subsequent effects to be exerted [see (41) for review]. Since many other substances, even D_2O in the medium (24), can produce similar effects, albeit usually at much higher concentrations than NGF, and some neuritogenesis can take place in culture medium even in the absence of added NGF, one is led to suspect that there are "many roads to Rome." If the rate-limiting step affected by NGF were known, it would be reasonable to expect that any treatment, chemical or physical, that would disinhibit it might have similar short- and long-range consequences for the responding cell.

It seemed reasonable to consider events that take place at the surface-water interface of NGF-responsive cells. It had been observed (29, 30) that, under particular circumstances, removal of protons not in instantaneous equilibrium with an unbuffered suspending fluid is required for Na^+-dependent uptake of γ-aminobutyric acid (GABA) to take place into mouse brain microsomal particles (P_3). This suggested that many other cellular processes may be regulated similarly. There is little doubt that the proton economy of the cell surface may play an extremely important role in regulating many aspects of membrane functionality because most enzymes, transporters, ion channels, and receptors show *p*H dependency. Indeed, through regulation of reactivities and conformations of membrane proteins

and their interactions with membrane phospholipids, protons may serve as on–off switches for many important membrane functions. Protons may be attached to membranes held in their close vicinity by strong coulombic interactions in unstirred electrical double layers, formed possibly with head groups of anionic phospholipids and/or with glycolipids and glycoproteins. The *p*H at the surface of a cell may not be the same as that of the fluid that bathes it. Not only are blood, lymph, ascitic fluid, and some culture media relatively poorly buffered, but also there may be regions on cell surfaces, such as deep invaginations, in which the extracellular fluid may not be in ready equilibrium with the bulk phase fluid. In addition, there would be differing rates of metabolic production and utilization of protons in different cell regions. For example, one might expect a greater tendency for acidification of the membrane (lower *p*H) in the vicinity of lysosomes than elsewhere.

We reasoned that a search for the presumed rate-limiting reaction in neuritogenesis would be facilitated if the pertinent experiments were conducted with sufficiently high concentrations of proton scavengers so that most, if not all, membrane regions of the cells under study actually would be at the *p*H of the medium, usually 7.3. This could be achieved by including in the medium adequate concentrations of appropriately chosen buffers. This would eliminate a whole set of variables that might be attributable to uncontrolled differences in local membrane *p*H and would enable more meaningful comparisons to be made between individual cells in which neuritogenesis can occur, such as rat pheochromocytoma PC12 cells, and rather tightly packed communities of cells in which neural and nonneural cells coexist, such as chick embryo ganglia. Differences between cells in the presence of proton scavengers and those under the "usual" culture conditions then might simplify identification of rate-limiting events that are affected by membrane *p*H and thus contribute to their further analysis. This study was the first step in that direction.

MATERIALS AND METHODS

Buffers and GABA Uptake. The buffers chosen for this study were triethanolamine (TREA), tris(hydroxymethyl)aminomethane (Tris), and N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid (Hepes). All three are widely used in biological studies. Their effects on uptake of GABA by a mouse brain microsomal fraction (P_3) were studied exactly as described elsewhere (29, 30). Unnecessary exposure of buffered culture media to strong light was avoided because of the recently discovered possibility of phototoxicity of such media (36).

Ganglia Culture. Dorsal root (DRG), trigeminal, and sympathetic ganglia from 7- to 8-day chick embryos were dissected in Dulbecco's phosphate-buffered saline (GIBCO), and were cultured in the presence of different concentrations of TREA, Hepes, and Tris buffers. In these preliminary experiments, we determined that concentrations between 2.5 and 5 m*M* of each buffer stimulated the maximal response of neurite extension in each instance (see below). Thereafter, DRG were used exclusively for the remainder of the experiments. Six DRGs were placed in each 60-mm culture dish (Falcon 3002). In the normal culture condition series, 5 ml complete medium was added to each dish. This medium contained 85% Dulbecco's modified Eagles' medium, 10% dialyzed fetal bovine serum (both from GIBCO), 3% glucose (600 mg/100 ml), 1% glutamine (200 m*M*), and 1% penicillin–streptomycin mix (GIBCO). Each set of experiments, repeated three or four times, consisted of two to four dishes per treatment. All dishes were incubated 3 days at 39°C in an air–5% CO_2 atmosphere in a water-jacketed incubator. In each experiment, a control group (untreated) and a group treated with 2.5s NGF (prepared by R. Bradshaw, UC Irvine, Calif.) at a final concentration of 10 n*M* were tested in parallel with the buffer-treated groups. Each buffer was prepared as a 10× stock solution. The buffers were constituted in phosphate-buffered saline with 2 m*M* $CaCl_2$, *p*H 7, at concentrations of 50 m*M*. and then were diluted in complete medium and the *p*H adjusted to 7.3. In every series, TREA, Hepes, and Tris were tested at final concentrations of 2.5 and 5.0 m*M*. A series of ganglia also was studied in the presence of cytosine arabinoside (ara C; Cytosar, Upjohn) at a final concentration of 8 μg/ml in the complete medium. The various buffers or NGF were added or direct current was applied to the DRG in ara C-containing medium.

With cycloheximide, the ganglia initially were incubated 3 h in complete medium containing 10 μg/ml cycloheximide (Sigma) and then were transferred to new culture dishes in complete medium and were subjected to the several treatments. Direct current was applied to the ganglia as described elsewhere (31). Electrically treated cultures contained 5 ml of the medium. The current was delivered through tantalum electrodes inserted in specially designed culture dish tops and connected to a 1.4-V battery. Constant currents of 10 nA were attained within 1 h and flowed for the entire 3-day period of the experiment.

PC12 Cell Culture. Rat pheochromocytoma cells (PC12) were grown routinely without antibiotics on uncoated Corning plastic tissue culture dishes using RPMI 1640 medium containing 4 m*M* glutamine, 10% horse serum, and 5% calf serum at 37°C in humidified air containing 3% CO_2. The sera were not heat-inactivated. Prior to subculture, the cell layers were rinsed with Ca^{2+}- and Mg^{2+}-free Hanks's saline, pipetted off the dish with

fresh serum-containing medium, and 1×10^5 cells were transferred to polylysine-coated dishes. One day later the medium was removed and serum-containing or serum-free medium with or without test substances was added. When the serum-free medium was used, the cells were subjected to a rinse with Hanks's solution. The serum-free medium consisted of RMPI 1640 with the following adducts: nonessential amino acids, 0.1 m*M* (GIBCO); ascorbic acid, 5 μg/ml; linoleic acid, 84 ng/ml; hydrocortisone, 18 ng/ml; insulin, 10 μg/ml; choline chloride, 9 μg/ml; vitamin B_{12}, 1.5 μg/ml; and fetuin, 1 mg/ml. Concentrated solutions of TREA, Hepes, Tris, and ara C were made in glass-distilled water at *p*H 7.4. NGF was dissolved in RMPI 1640 with 5% calf serum. Prior to use all solutions were filtered through 0.45-nm, detergent-free filters. The cultures were studied after 4 to 6 days exposure to the test substances and compared with suitable controls. RMPI 1640, Hanks's saline, and sera were obtained from Irvine Scientific Company in Irvine, California, NGF from Collaborative Research in Waltham, Massachusetts, and other substances from reliable commercial sources.

Fixation, Staining, and Radioautography. At the end of incubation, the cultured ganglia or PC12 cells were fixed in 3.5% glutaraldehyde in 0.1 *M* cacodylate buffer at room temperature for 2 h and the ganglion cultures were stained with toluidine blue. In some instances the cultures were washed twice at 4°C in 0.1 *M* cacodylate buffer after glutaraldehyde and the preparations stained with Cajal's silver stain. The PC12 cultures were viewed under phase contrast for evaluation. For radioautography of the ganglia, [^{3}H]proline (L-2,3-[^{3}H]proline; 40 Ci/m*M*) was added to the cultures at a final radioactivity of 4 μCi/ml 20 h prior to fixation. After fixation with glutaraldehyde and three washes with 0.1 *M* cacodylate buffer, the culture dishes were coated with NTB2 liquid emulsion (Kodak) in the dark, and the dishes drained, inverted, and air-dried. Exposure took place at 4°C for 3 weeks in light-tight boxes and the radioautographs were developed at 18°C in Dektol, fixed, washed several times in water, and dried.

Neurite Outgrowth. Neurite outgrowth in the ganglia was determined after incubation according to the method of Fenton (11). This method assigns a score to each ganglion based on the number, length, and degree of branching of the neurites emanating from the original explant. The scoring system assigns 0 to a ganglion with no outgrowth and +5 to a ganglion with maximal outgrowth. Treatment with 10^{-8} *M* 2.5s NGF routinely yielded close to a +5 score. To compare treatments, scores for all ganglia in their respective groups were pooled and means and standard deviations were obtained. Using NGF, most observers note the maximal response after 1 or 2 days *in vitro*, the response being somewhat more variable at 3 days. However, we have used this later time in experiments in

which we determined long-term effects with a Cajal silver stain to substantiate phase microscopic scoring of the neurite outgrowth. Dunnett's test was applied as a test of significance. It is a multicomparison of treatment means with a control (P limit = 0.01). Only qualitative observations and semiquantitative estimates were made in the case of the PC12 4 to 6 days after exposure to test substances.

RESULTS

Effects of Triethanolamine, Tris, and Hepes on GABA Uptake. TREA, Tris, and Hepes all enhanced the uptake of GABA by the P_3 particles as a function of their concentrations (Fig. 1). On a molar basis, TREA was the most effective substance of the three. The maximal effects of all the buffers were exerted at approximately 5×10^{-2} M, and somewhat lesser effects were observed at 2.5×10^{-2} M. Decrements from the maximal effects were observed when concentrations of the buffers exceeded 1×10^{-3} M. In all subsequent experiments, the effects of the above substances were tested at 2.5 and 5.0 mM concentrations, respectively.

Effects of Triethanolamine, Tris, Hepes, and Cytosine Arabinoside on Neuritogenesis in Chick Embryo Ganglia. In the first experiments the various peripheral ganglia were cultured in the presence of concentrations of TREA from 1 to 10 mM. We determined that trigeminal, dorsal root, and sympathetic ganglia all responded by elaborating neuritic processes at concentrations between 2.5 and 5 mM, and adverse effects often were observed at higher concentrations. In contrast to trigeminal ganglia in control medium, trigeminal ganglia cultured in 2.5 or 5 mM TREA and sympathetic ganglion cultured in 2.5 mM TREA all demonstrated long, filamentous, neuritic processes that extended from the centrally neuronal cell bodies out to and beyond the mat of underlying nonneuronal cells (Fig. 2). Preliminary semiquantitative assessment of neurite outgrowth was determined in the series of experiments conducted on DRGs in complete

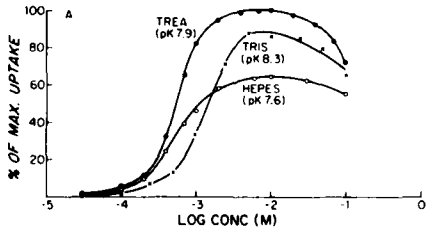

FIG. 1. Concentration dependence of enhancement of GABA uptake into mouse brain P_3 particles by triethanolamine (TREA), Tris, and Hepes.

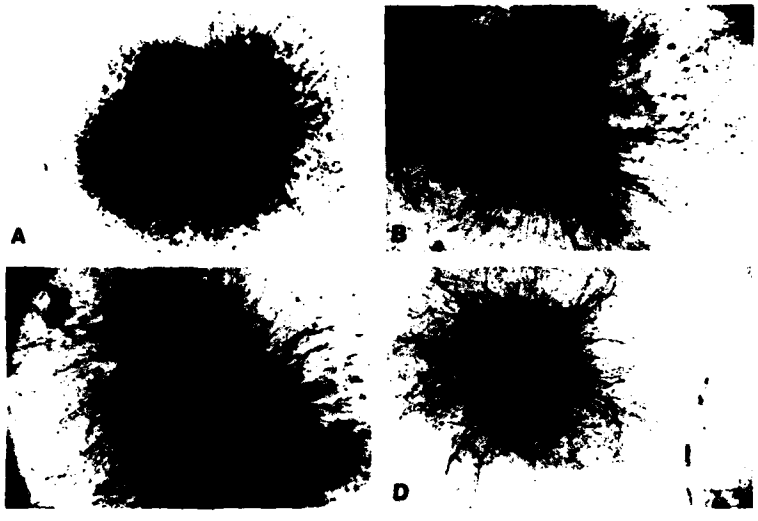

FIG. 2. Trigeminal and lumbar sympathetic chain ganglia cultured in complete media 3 days *in vitro*. Control culture of trigeminal ganglia contained few neurites (A); trigeminal ganglia in 5 m*M* TREA (B) or 2.5 m*M* TREA (C) contained long neuritic processes emanating from the cell bodies in the center of the ganglia. Enhanced neurite outgrowth was also seen in sympathetic ganglia cultured in 2.5 m*M* TREA (D). All ganglia stained with Cajal silver stain. × 30.

medium. The average scoring of the responses in these cultures was: control, +1.5; NGF, +4.4; 5 m*M* TREA, +3.4. Qualitatively similar results were obtained with DRG upon treatment with NGF, TREA, or electric current (Fig. 3).

The quantitative data from the entire series are summarized in Table 1. The short preincubation (3 h) with 10 μg/ml cycloheximide appeared to have no effect or to slightly enhance neurite extension in the various treatment groups over that found under normal conditions of culture [see also (22)]. Histological observations indicated that cycloheximide diminished the nonneural population so that the neurons were visualized more prominently. In addition, many more neural cells had migrated out of the central portions of the ganglia. Radioautographic evidence (not shown) of inhibition of protein synthesis by cycloheximide during neurite extension was obtained in all instances using [^{3}H]proline as label. Thus, the experiments with cycloheximide-treated ganglia showed that the effects of the substances tested and direct current on neuritic extension do not appear to require protein synthesis to be taking place.

Constant incubation in the presence of ara C increased neuritogenesis in otherwise untreated cultures relative to that observed in its absence (Table

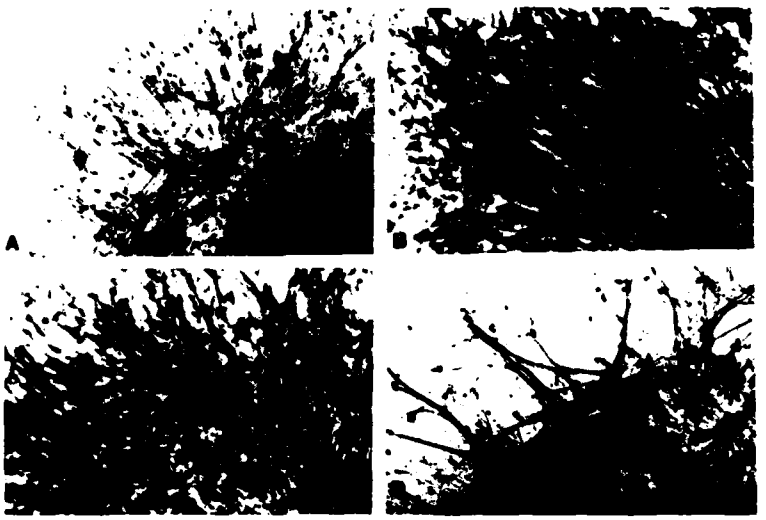

FIG. 3. Dorsal root ganglia grown 3 days in complete media to demonstrate differential effects of the various treatments. Control (A); 10 n*M* NGF (B); 10 nA direct current (C); 5 m*M* TREA (D). Cajal silver stain. × 78.

1). Although the population of nonneural cells was markedly depleted by ara C, there appears to have been no effect or a slight enhancement of neuritogenesis in the presence of TREA or when DC was applied, but there was a slight decrement in the presence of NGF. Similar results were observed (33) in simultaneously conducted experiments on the effects of pulsed electromagnetic fields and direct current. Radioautographs of ganglia treated with ara C provided confirmatory evidence for those results (Fig. 4). Although radioactivity was found in the nonneuronal cells that remained, incorporation of [^{3}H]proline into neuronal cell bodies and their processes was easily visualized. The neurites in these preparations were easily distinguishable and often could be traced back to the parent cell body. Obviously, ara C not only had not interfered with neuritogenesis, but actually had enhanced it.

Nerve growth factor but not Tris, TREA, or Hepes stimulated an extension of neurites in PC12 cells (Fig. 5). Results are shown in Fig. 5 only for TREA, and not for Tris or Hepes, which were similar. PC12 cells grew only in the presence of serum but were able to survive 5 to 7 days in the enriched serum-free medium described above. The cells had a tendency to flatten out on the surface of the polylysine-coated dishes and extended short, stubby processes which often gave the cells a stellate appearance (Fig.

TABLE 1

Neurite Extension in Chick Embryo Dorsal Root Ganglia

	Fiber index											
							Cytosine arabinoside					
	Normal culture			Cycloheximide			A			B		
Treatment	Mean (*N*)	SD	*P*[a]	Mean (*N*)	SD	*P*[a]	Mean (*N*)	SD	*P*[a]	Mean (*N*)	SD	*P*[a]
Control	1.53 (16)	0.74	—	2.0 (21)	1.4	—	3.10 (45)	0.72	—	3.95 (11)	0.62	—
NGF (10^{-8} *M*)	4.42 (19)	1.07	0.01	4.27 (18)	1.11	0.01	3.7 (23)	0.97	0.01	3.23 (10)	0.75	0.05
TREA												
2.5 m*M*	3.07 (28)	1.03	0.01	4.57 (14)	0.78	0.01						
5.0 m*M*	3.42 (32)	1.21	0.01	3.82 (20)	0.73	0.01	4.0 (34)	0.79	0.01			
Hepes												
2.5 m*M*	2.66 (18)	1.17	0.01	2.93 (7)	1.17	ns						
5.0 m*M*	3.18 (29)	0.89	0.01	4.19 (21)	0.98	0.05						
Tris												
2.5 m*M*	2.55 (17)	0.86	0.01	2.0 (13)	0.73	ns						
5.0 m*M*	2.55 (17)	0.61	0.01	3.52 (25)	0.99	0.01						
Direct current (10 nA)	2.71 (38)	0.13	0.01	3.97 (22)	0.82	0.01	3.42 (39)	0.6	ns	4.52 (9)	0.40	ns

[a] Dunnett's test: multicomparison of treatment means with a control (*P* limit = 0.01). In all instances comparisons of treatment groups were made with the specific controls for that group.

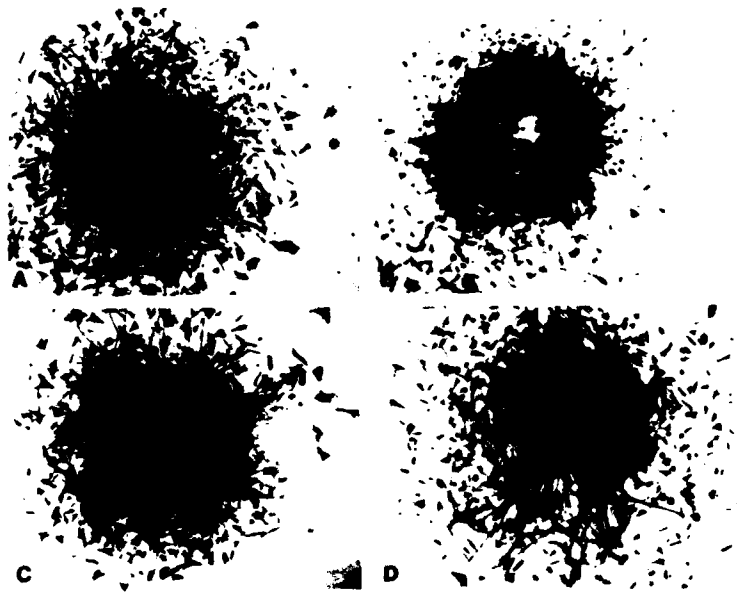

FIG. 4. Dorsal root ganglia grown 3 days in media containing 8 μg/ml cytosine arabinoside and incubated in [^{3}H]proline for the last 20 h to show incorporation into proteins on radioautographs. Neurons and neurites contrasted out sharply with the gray (flat) nonneuronal cells. Control (A); 10 n*M* NGF (B); 10 nA direct current (C); 5 m*M* TREA (D). × 42.

5A). There was less tendency for the cells to flatten in serum-free medium, but the short processes were present (Fig. 5D). NGF (50 ng/ml) caused appearance of neurites in both serum-containing and serum-free medium (Fig. 5B and E). In the latter (Fig. 5E), the neurites were thicker and more heavily beaded than in the serum-containing medium (Fig. 5B). In contrast to the chick DRG, PC12 cells did not respond with extension of neurites in the presence of TREA, Tris, or Hepes, but as illustrated for TREA (Figs. 5C and F) retained the appearance of control cells. However, in a preliminary study, TREA was found to enhance the growth of PC12 cells. The following average cell numbers for two closely checking dishes at each concentration were observed at 4 days of growth in complete medium at successively increasing concentrations of TREA when 1.07×10^5 cells were plated initially: control, 2.85×10^5; 2.5 m*M*, 4.48×10^5; 5.0 m*M*, 4.66×10^5; 7.5 m*M*, 4.76×10^5; and 10.0 m*M*, 6.01×10^5. TREA was toxic to the PC12 cells at 20 m*M*. Further work is in progress with TREA to substantiate these latter results and with other buffers, as well.

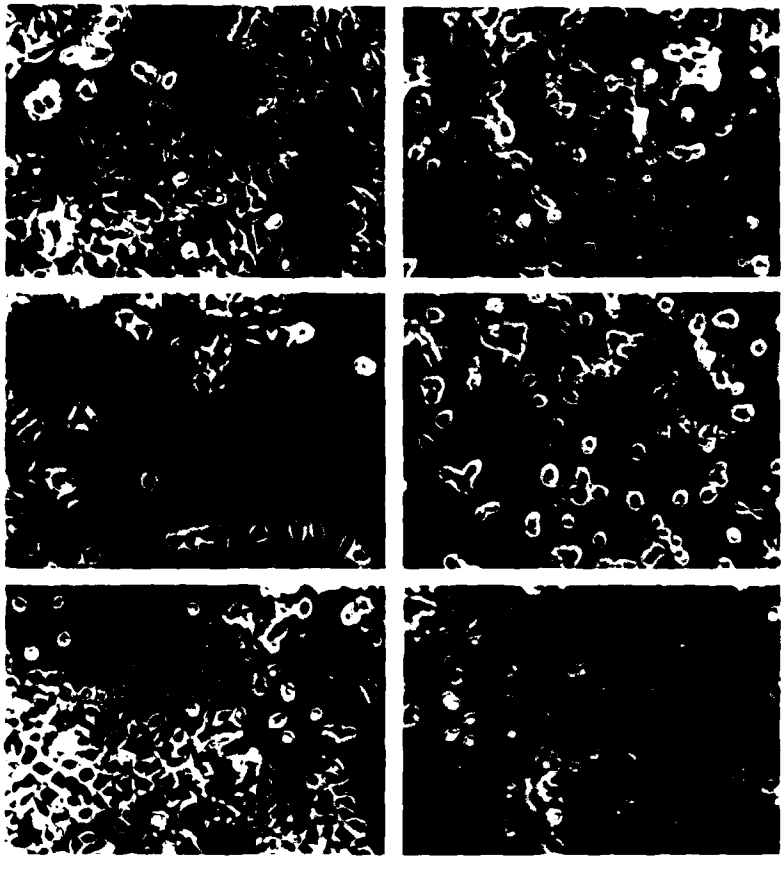

FIG. 5. Stimulation of neurite outgrowth in pheocytochromagen PC12 cells by NGF but not by TREA. Control in serum-containing (A) and serum-free medium (D); NGF (50 ng/ml) in serum-containing (B) and serum-free medium (E); TREA (5 m*M*) in serum-containing (C) and serum-free medium (F).

A series of experiments was carried out in the California laboratories with media which had been removed under sterile conditions from cultured trigeminal ganglia and DRGs after 4 days of culture in the Kentucky laboratories, were frozen, and shipped to California, where they were filtered prior to use. The media were either control media or those containing NGF or 5 m*M* TREA. PC12 cells were plated in serum-containing medium, as described, and subsequently for 6 days in medium of which 25% consisted

of the "conditioned" medium. Control PC12 cells were grown with no addition or with NGF (50 ng/ml). Media from ganglia in which TREA or passage of electric current had elicited outgrowth of neurites produced no observable effect on PC12 cells under our experimental conditions. Only the dishes containing cells which had been grown in conditioned medium with NGF or to which NGF had been added showed significant neuritogenesis. If there had been liberation of a neuritogenic factor into the medium in which ganglia had been grown, it either was ineffective on PC12 cells or was present in concentrations below an effective threshold.

DISCUSSION

Our original purpose was to determine whether or not neuritogenesis and other grossly observable characteristics of neural cells in culture could be affected by proton removers (buffers) added to the bicarbonate–CO_2 buffer already present in order to bring the cell surface *p*H close to that of the ambient medium. We used three buffers in concentrations judged to be sufficient to achieve that purpose, although direct measurements were not made of *p*H in the immediate neighborhood of the membranes themselves. In the future, such measurements are planned with lipid soluble indicators that can probe *p*H changes at inner and outer membrane surfaces (28).

In the concentrations used, 2.5 and 5.0 m*M*, all three buffers, TREA, Tris, and Hepes, enhanced neuritogenesis significantly in explanted chick embryo ganglia over that found in their absence, although the *p*H indicator in the medium showed the *p*H of the medium to be the same whether or not the above buffers were present. This is reminiscent of the results with the above three buffers and 18 other buffers on the enhancement of uptake of GABA by a mouse brain synaptosomal subfraction at 0°C and *p*H 7.3 (29, 30). We presume that in both instances the effects of the buffers were attributable to proton removal by the unprotonated forms of the buffers, as otherwise the substances show considerable structural diversity. For example, in the instance of the three substances used in the present study, TREA is a tertiary amine and Tris is a primary amine; in both, the unprotonated form is uncharged and the protonated form is cationic at *p*H 7.3. Hepes is a disubstituted piperazino derivative possessing a sulfonic acid group, both protonated and unprotonated forms being anionic at *p*H 7.3. The different efficacies in enhancement of GABA uptake (Fig. 1) may be attributable, in part, to the effect of structural differences on interactions with membranes and possibly on the entry of the buffer into them and passage through them into the cell interior (29, 30). Nonetheless, all three buffers proved to be neuritogenic in the absence of added NGF in our test system in concentrations to 5 m*M*. Adverse effects on fiber outgrowth were observed at 7.5

m*M* and above. It is interesting in this regard that *N*-[tris(hydroxymethyl)methyl]glycine and Hepes were found to be nontoxic for chick embryo DRGs at 50 m*M* when used to maintain *p*H in the absence of bicarbonate–CO_2, but no fiber outgrowth was observed at that concentration of buffer in the absence of added NGF (16).

A key finding in our study was that neuritogenesis, which occurs to a slight extent in ganglia maintained under the usual conditions and is greatly stimulated by NGF, was also enhanced by such divergent treatments as addition of buffer, the passage of electrical current, and the presence of ara C in the medium. From radioautographic observations and the failure of cycloheximide to eliminate the above neuritogenic effects and from the experiments with ara C, it is apparent that DNA and protein synthesis are not required for the production of neurites by neural cells in the ganglia under our conditions. One possibility is that the above, and other treatments (1, 2, 5–7, 10, 17, 20, 26, 37), enable the release of growth substances to take place from the nonneural cells to neural cells, thus evoking neuritogenesis in the latter. It will be possible to test whether or not one of these is chick NGF by determining if its effects could be eliminated by application of specific antiserum to it, when such an antiserum becomes available. Similar strategies could be applied to other growth factors, as they become known. However, the lack of effect on PC12 cells of media in which neuritogenesis in ganglia had been elicited by TREA does not support the hypothesis that NGF, itself, or some other potent neuritogenic factor might be liberated into the medium. Of course, exchange of materials between neurons and nonneural cells could take place through gap junctions between them without such materials ever appearing in the bulk fluid surrounding the cells. Appropriate immunocytochemical experiments would be required to settle such an issue. Obviously, the experiments carried out to date are only preliminary and much more work needs to be done.

In our experiments NGF was effective in eliciting neuritogenesis both in explanted chick embryo ganglia and in pheochromocytoma PC12 cells. The buffers were effective only on the ganglia. Although experiments are planned with neuron-enriched preparations from dissociated ganglia to determine whether or not the buffers can exert direct effects on the neurons, currently it seems that an indirect effect is more likely. The removal by buffers of protons from the region of the neural membrane may sensitize the membrane to depolarizing influences which are present in the medium, possibly in the serum (40), which increase the influx of Na^+ and Ca^{2+}. One consequence of the latter might be a release of transmitter-like substances and K^+ from the depolarized neurons. The latter ion may be an important signal transmitted from neurons to glia and possibly to other nonneural cells immediately in the vicinity. The glial cells, acting essentially like potassium

electrodes, would become depolarized (27) in turn, and the consequential membrane changes, including intracellular calcium liberation and membrane fluidization, could result in release from them and transmittal to neural cells of NGF and a variety of other neuronotrophic factors. In the absence of sufficient nerve activity in the ganglia in culture, release of such substances from glial cells might not take place. Relevant to the above is the finding that nanoampere levels of direct current applied *in vitro* to trigeminal, dorsal root, and sympathetic chain ganglia from 8-day chick embryos stimulate cathodically oriented neurite outgrowth, increase neuronal survival and differentiation, and enhance metabolite uptake (32). In this regard, it is of great interest that one of the early important effects of NGF is on ionic responses in sensitive cells (3, 4, 18, 19, 34, 35).

Another possibility is that the capacity of neurons in the ganglia to produce neurites is inhibited under the usual conditions of culture, as if the neural cells are held in a "straight-jacket," possibly both physically in a tightly packed environment and by the liberation of inhibitory substances from the nonneural cells in the ganglia. A loosening of the straight-jacket by allowing cell migration to take place coupled with decreases in the numbers of the nonneural cells and/or decreases in their metabolic activities may then allow the neurons to express an inherently residing neuritogenic potential. Although such inhibitory substances have not yet been found, there is little evidence that they have been seriously sought. If the state of activation of the neurons in the ganglia at any particular time is a resultant of the effect of inhibitory and stimulatory influences exerted on them largely from nonneural cells in the ganglia, then a net decrease in inhibitory influences, such as might be consequent to the action of ara C, might enhance the probability of their survival and differentiation.

For those interested in the "sociology" of cellular interrelationships, the challenge is to elucidate the patterns of communications between the different cell types, their evolution during development, and the effects on them of hormones, drugs, and a variety of environmental factors. Conversion of closed looped systems (cybernetically effective) to open looped ones (cybernetically ineffective) can lead to growth or to pathologic manifestations at intracellular, cellular, and organismic levels. Perhaps the development of chemical and physical means for manipulating relationships between cells in chick embryo ganglia and the understanding which that may bring, eventually will lead to the devisal of new strategies for alleviating inadequate intercellular communication, wherever it is found to occur. It was, therefore, decided to test the effects of two of the substances that were found to be neuritogenic in the present study, TREA and ara C, in a situation in which such incoordination is known to exist, the injured vertebrate spinal cord (13), as will be illustrated in the following paper.

Those working with NGF should be grateful that Tris, TREA, and Hepes were not used in culture media at the time that work on this factor was begun. If these substances had been used as buffers in the culture media used originally, the neurite extension assay might not have given such clear-cut results as it did, and the cumbersome and less sensitive assays with whole chick embryos might have greatly retarded progress on the purification and characterization of NGF.

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PEMF, DIRECT CURRENT AND NEURONAL REGENERATION: EFFECT OF FIELD GEOMETRY AND CURRENT DENSITY

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ABSTRACT

Sensory ganglia from 7-8 day chick embryos were exposed to pulsed electromagnetic fields (PEMF) or direct current(DC) in order to correlate stimulation of neurite outgrowth with current density as a function of field geometry. Growth scores were obtained on ganglia growing in the inner and outer rings of 50 mm culture dishes. Control cultures and cultures treated with nerve growth factor served as standards. In PEMF experiments with the coil pair oriented horizontally, no correlation was observed between ganglia growth and current density in contrast to our previous findings with the coils oriented vertically. Comparison of current density for vertical and horizontal coils driven identically suggests a dose-saturation effect for the induced current with a threshold at approximately 0.4 uA/cm^2. Application of DC elicited significantly greater growth as a function of location with current density levels above 9 nA/cm^2. Interestingly, the total charge input for PEMF and DC stimulation was nearly identical, 10^{-3} coulombs.

INTRODUCTION

Externally-applied electric fields have been used to stimulate nerve regeneration and function (5,6,8,10,11,15,16,17,21-28). These studies have been carried out on both central and peripheral nerves, *in vivo* and *in vitro*, testing direct current (DC) applied with agar or metal electrodes, and current induced by pulsed electromagnetic fields (PEMF).

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In our laboratory, we have addressed the question of neuronal growth stimulation using either DC or PEMF. Direct current has been applied *in vitro* to embryonic sensory neurons with agar salt bridges (23), or metal electrodes (22-26). Three different waveforms generated from Helmholtz coils (ElectroBiology, Inc, NJ) designed to test varied frequency content (12,13)have been studied in this system; only the single pulse 72 Hz signal elicited significant stimulation of growth.

In our previous paper (25), we reported on the effects of direct current and the single pulse PEMF on cultures of chick dorsal root ganglia. The PEMF was applied to the cultures with the coils oriented vertically. In this orientation the level of current density, which is dependent upon the height of the medium, is higher in the center of the dish relative to that in the periphery of the dish. Significant stimulation of growth was correlated with the higher current density (0.7 uA/cm^2)in the dish center. In this paper we will examine the effects of PEMF when applied with the coils oriented horizontally to continue the dose-effect study and determine if there is also a correlation of growth with location and current density.

MATERIALS AND METHODS

Dorsal root ganglia (DRG) from 7-8 day chick embryos were dissected in Dulbecco's phosphate buffered saline (Gibco Co.), and 6-8 DRG were placed in 5 or 8 ml culture medium in Falcon 3006 culture dishes with liners. Two types of culture media were used: complete medium consisting of Dulbecco's Modified Eagles's Medium with 10% dialyzed fetal calf serum (Gibco), 600 mg% glucose, glutamine (2mM) and 1% penicillin/streptomycin, or complete medium containing cytosine arabinoside(ara C, Cytarabine, Upjohn) at a final concentration of 8 ug/ml.

In most cases, four treatment groups containing four dishes per group were run on each day's experiment. The groups were: a control,

non-treated group; a group treated with 10 nM nerve growth factor (NGF); a group treated with pulsed electromagnetic fields (PEMF) for 12 hours per day for two days(24 hours total exposure); and a group treated with direct current continuously for three days. Each experiment was repeated 3-4 times.

Nerve growth factor (7s) was obtained from The Research Foundation of State University of New York, Albany, N.Y.

The pulsed electromagnetic fields (PEMF) were generated by research coils provided by ElectroBiology, Inc., N.J. A single pulse waveform (Figure 1a) was used. The dishes were placed in a horizontal orientation; i.e., parallel to the coils (Figure 1b). In this orientation, the current density at the center of the dish is zero and increases to a maximum of 4.7 uA/cm^2 at the edges of the dish (Figure 2). The distribution or magnitude of current density is independent of the volume of medium in the dish in contrast to dishes placed between vertically-oriented coils (9,14,25). The spatial distribution of the magnetic field is nearly constant across the dish. The time rate of change of the magnetic field during the main polarity pulse is 5.3 Tesla/sec.

Direct current was administered to the cultures by connecting two tantalum electrodes immersed in the medium to a 1.4 V battery. A non-uniform field was created between a single center cathode and a circular peripheral anode (22). The total current delivered in 8 ml medium was 10 nA/dish. The electric field (E) and current density (J) in this volume is depicted in Figure 3. The calculations used to determine these were: $E = I \times R/2\pi r \times h$, and $J = E / R$ (I = total current; R = 71 Ohm cm; r = radius; h = height of media).

All dishes were incubated in 5% CO_2, 95% air in a water-jacketed tissue culture incubator for 6 days. To determine protein synthetic activity, two dishes from each group were exposed to ^{3}H-proline at a final concentration of 10 uC/ml for the last 20 hours of incubation. They were then fixed, as were all other cultures, with 3.5% glutaraldehyde in 0.1 M cacodylate buffer and scored for neurite

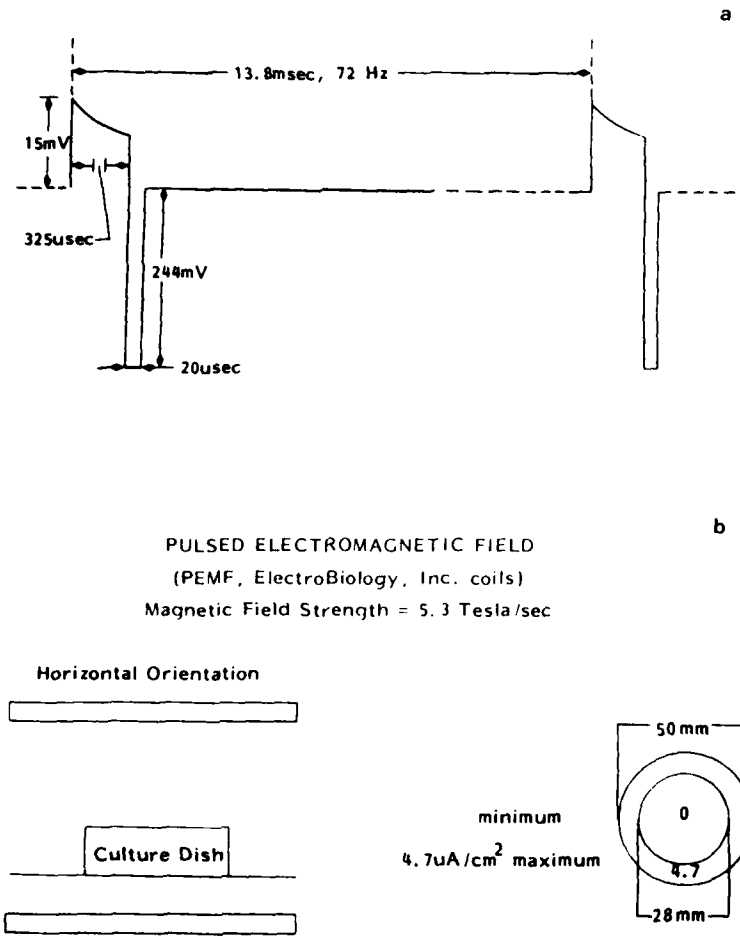


FIGURE 1

(a) Pulse waveform generated by Helmholtz coils. (b) Placement of the culture dishes between the horizontally-oriented Helmholtz coils (left), and levels of current density within each culture dish (right).

FIGURE 2

Current Density (uA/cm^2) as a function of dish radius, independent of height of the medium (PEMF).

outgrowth. The cultures exposed to the ^{3}H-proline were subsequently rinsed in buffer 3 times, then with distilled water, drained and covered with Kodak NTB2 liquid emulsion. They were exposed for 3 weeks in light-tight boxes at 4°C and developed in Kodak Dektol(1):water(2) and fixed in Kodak Rapid Fix. Photographs were taken on a Zeiss microscope with light and phase optics using Kodak Plus X film.

The determination of neurite outgrowth was based on a standard scoring system of 0-+5 max which involves the number and distribution of neurites (axons/dendrites) emanating from the centrally-placed neuronal cell bodies using a standard nerve growth factor preparation (24). To correlate neurite outgrowth with position of the ganglia in the dish, and therefore, current density, a transparent guide

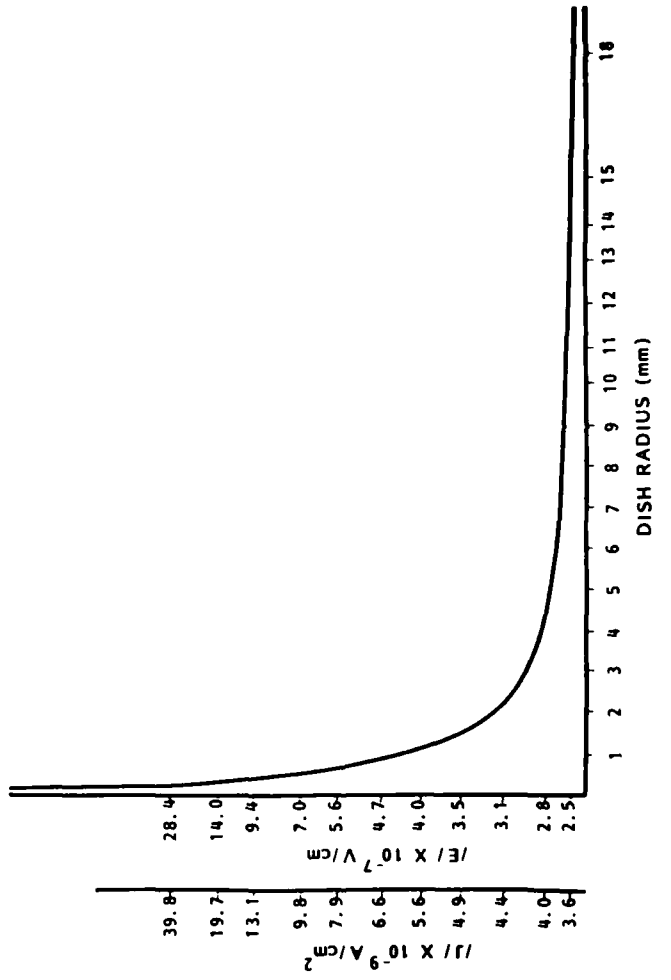


FIGURE 3

Electric Field (E) and current density (J) on bottom of dish containing 8 ml medium in (DC).

containing concentric rings was placed under the bottom of the culture dish. The total diameter of the dish bottom was 50 mm; the inner ring had a radius of 14 mm., the outer ring, 11 mm. (see Figure 1b). The position of each ganglia in a dish was noted and the neurite outgrowth was determined. The paired t test for related measures was used to determine significance of growth relative to location; only dishes containing ganglia in both inner and outer rings were used in these analyses. Significant differences between the mean of the control group and the means of the various treatment groups were determined by the Dunnett's Multiple Comparison Test (significance of p=.01).

RESULTS

In PEMF-treated dishes containing either 5 or 8 ml medium, the current density in the inner ring varies from zero to 2.65 uA/cm^2 (av. 1.32), while that in the outer ring varies from 2.65 to 4.7 uA/cm^2. Since most of the ganglia scored in the outer ring were found between 14 and 24 mm radius location (2.65-4.5 uA/cm^2), the average current density exposure was 3.57 uA/cm^2.

The current density in the direct current-treated dishes (8 ml volume) in the inner ring varied from 39.8 nA/cm^2 at 1 mm from the cathode to 2.78 nA/cm^2 at 14 mm. Most of the drop in current density occurred within the first 3 mm where less than 5% of the ganglia were found; the current density from 3 mm to 14 mm averaged 5.76 nA/cm^2. The current density in the outer ring was fairly constant, varying from 2.78 -2.19 nA/cm^2 (Figure 3). Tables 1 and 2 contain data found in cultures of dorsal root ganglia grown in 5 ml complete media, or in 5 ml complete media plus ara C. Only control and PEMF groups were run in this series; we have already presented data on the other two groups (25). In both tables, the mean score of all ganglia per dish was determined, and the mean and standard deviation of dish scores in each group was calculated.

In complete medium, 56 ganglia in 13 dishes treated with PEMF did

TABLE 1

Dorsal Root Ganglia in 5 ml Complete Medium
Comparison of Neurite Outgrowth Between Groups*

Group	Number Dishes	Number Ganglia	Mean Score ± SD	p
Control	12	46	1.93 ± .83	
PEMF, H	13	56	2.13 ± .72	ns
	25	102		

TABLE 2

Dorsal Root Ganglia in 5 ml complete Medium + 8 ug/ml ara C
Comparison of Neurite Outgrowth Between Groups*

Group	Number Dishes	Number Ganglia	Mean Score ± SD	p
Control	8	32	4.13 ± .26	
PEMF, H	8	45	3.97 ± .52	ns
	16	77		

*Significance determined by Student's t test

not exhibit significantly greater neurite outgrowth than 46 ganglia in 12 control dishes (Table 1). In ara C medium, the scores of both groups were significantly higher, but there was no difference noted between 8 control and 8 PEMF dishes. Morphological evidence for the increase in growth scores, when the ganglia are incubated in ara C medium, has been noted before (23,25), and occurs also in 8 ml medium (Figure 5).

To determine whether there were any significant differences between growth and location of the ganglia in the dish, ganglia in the inner ring were compared to those in the outer ring. In either complete medium (Table 3), or in ara C medium (Table 4), no differences were noted.

Four groups were tested in cultures grown in 8 ml of medium. The mean scores of these groups, in complete medium, are presented in Table 5. In 16 dishes, the score of the control group was less than that obtained in the control group grown in 5 ml medium, while the

TABLE 3

Dorsal Root Ganglia in 5 ml Complete Medium
Neurite Outgrowth Relative to Location in Dish*

		Outer Ring		Inner Ring		
Group	Number Dishes	Number Ganglia	Mean Score ± SD	Number Ganglia	Mean Score ± SD	p
Control	9	12	1.99 ± 1.04	24	1.7 ± .98	ns
PEMF, H	8	13	2.17 ± .52	27	1.83 ± .95	ns
	17	25				

TABLE 4

Dorsal Root Ganglia in 5 ml Complete Medium + 8 ug/ml ara C
Neurite Outgrowth Relative to Location in Dish*

		Outer Ring		Inner Ring		
Group	Number Dishes	Number Ganglia	Mean Score ± SD	Number Ganglia	Mean Score ± SD	p
Control	5	13	3.95 ± .37	14	4.04 ± .3	ns
PEMF, H	8	27	3.85 ± .5	18	3.7 ± .82	ns
	13	40				

*Significance determined by paired t test

TABLE 5

Dorsal Root Ganglia in 8 ml Complete Medium
Comparison of Neurite Outgrowth Between Groups*

Group	Number Dishes	Number Ganglia	Mean Score ± SD	p
Control	16	73	1.28 ± .49	
NGF	11	67	2.85 ± .64	sig
PEMF, H	16	69	2.16 ± .76	sig
DC	14	60	2.60 ± .47	sig
	57	269		

*Significance determined by Dunnett's Multiple Comparison Test (p=.01)

score in the PEMF group was unchanged. PEMF, NGF and DC treatments all stimulated significantly greater neurite outgrowth than that of the control group, with NGF eliciting the greatest response. Typical growth patterns in all groups are illustrated in radioautographs of incorporated ^{3}H-proline in Figure 4.

Neurite outgrowth scores of ganglia grown in 8 ml ara C medium (Table 6) are higher than those in complete medium in all groups with the exception of NGF, which is significantly lower than the control group. No significant differences were found between control and PEMF or DC groups. Figure 5 demonstrates the increased neurite outgrowth in Control, PEMF and DC, and the diminished response in the NGF cultures.

Comparisons of growth scores between the inner and outer rings in these groups are presented in Tables 7 and 8. In 50 dishes containing 245 ganglia grown in 8 ml complete medium distributed among the four groups, there were no significant differences in neurite outgrowth correlated with location. Particular attention was paid to ganglia located in the exact center of the dish in the PEMF group (zero current density); growth patterns here were comparable to those found throughout the dish, confirming the lack of correlation of growth with current density.

Data obtained from dishes containing 174 ganglia from 35 cultures grown in 8 ml ara C medium are presented in Table 8. Only in the NGF group was any difference noted between ganglia located in inner and outer rings, and this was not significant at the .01 level.

DISCUSSION

This study has investigated the question of whether there is a correlation between the amount of current density induced by pulsed electromagnetic fields, or direct current, and the degree of stimulation of neurite outgrowth. We have addressed this question by determining the current density in the dish as a function of radius. Control and nerve growth factor preparations served as standards for

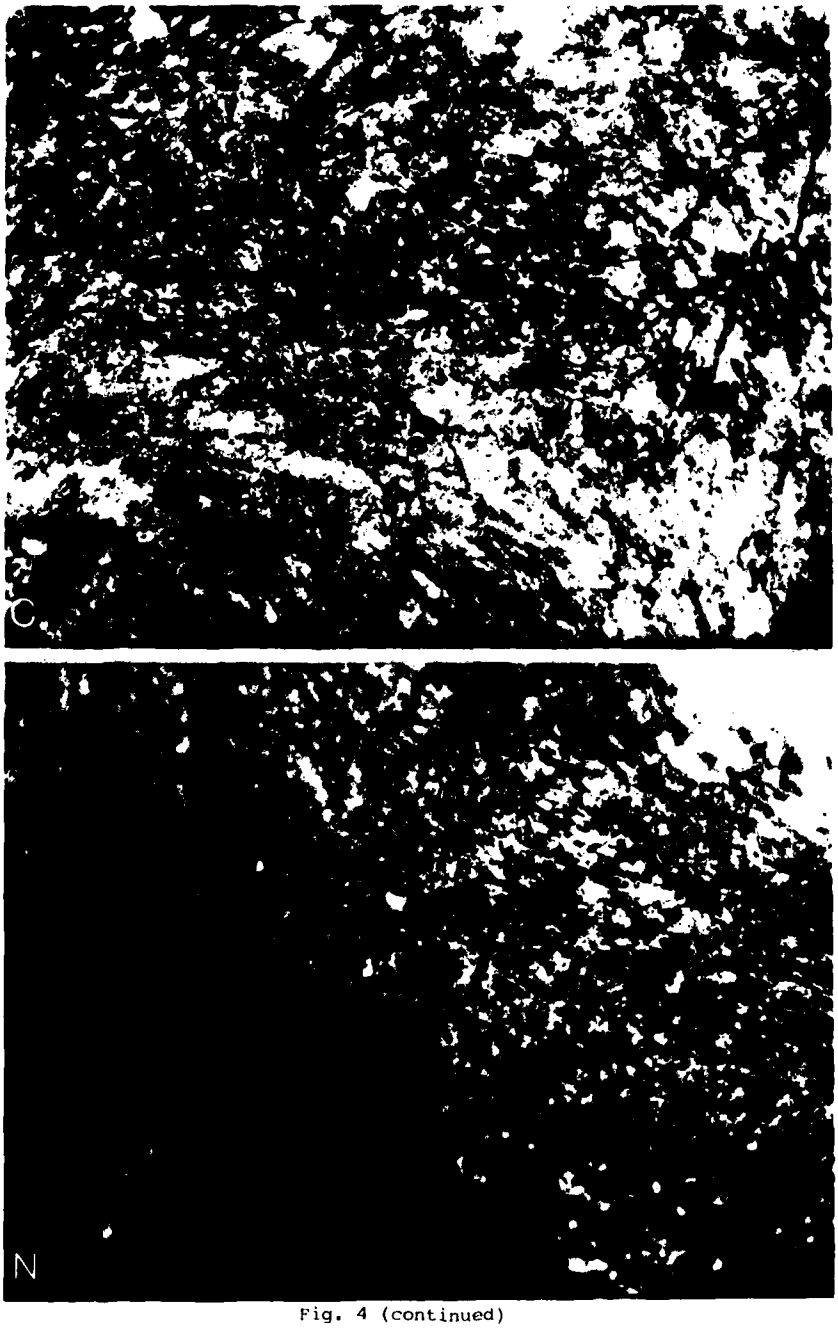


Fig. 4 (continued)

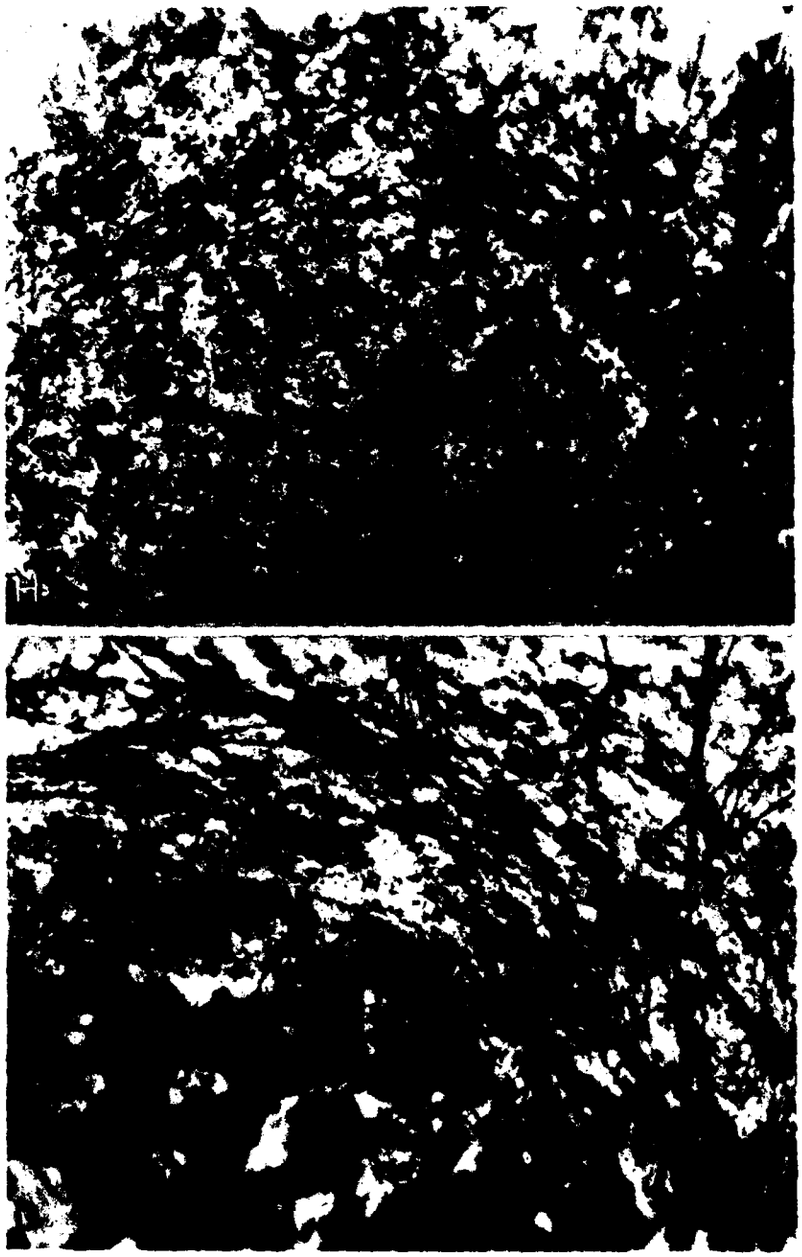


TABLE 6

Dorsal Root Ganglia in 8 ml Complete Medium + ug/ml ara C
Comparison of Neurite Outgrowth Between Groups*

Group	Number Dishes	Number Ganglia	Mean Score ± SD	p
Control	10	48	2.58 ± .42	
NGF	10	37	1.89 ± .58	ns
PEMF, H	12	55	3.40 ± .42	ns
DC	11	51	3.39 ± .39	ns
	43	191		

*Significance determined by Dunnett's Multiple Comparison Test (p=.01)

TABLE 7

Dorsal Root Ganglia in 8 ml Complete Medium
Neurite Outgrowth Relative to Location in Dish*

	Outer Ring			Inner Ring		
Group	Number Dishes	Number Ganglia	Mean Score ± SD	Number Ganglia	Mean Score ± SD	p
Control	15	39	1.25 ± .47	30	1.39 ± 1.06	ns
NGF	11	32	2.75 ± .59	35	2.88 ± .8	ns
PEMF, H	11	23	2.19 ±1.02	29	2.22 ± 1.13	ns
DC	13	21	2.70 ± .7	36	2.68 ± .75	ns
	50	115		130		

FIGURE 4

Dorsal root ganglia after 6 days in vitro in 8 ml medium. Radioautographs of whole explants after incubation in ^{3}H-proline for the last 20 hours of incubation. Control (C); NGF (N); PEMF horizontal (H); and Direct Current (D). Neuronal cell bodies in the center of the explant, and neurites contain great numbers of grains so that they appear as black silhouettes. X192.

TABLE 8

Dorsal Root Ganglia in 8 ml Complete Medium + 8 ug/ml ara C Neurite Outgrowth Relative to Location in Dish*

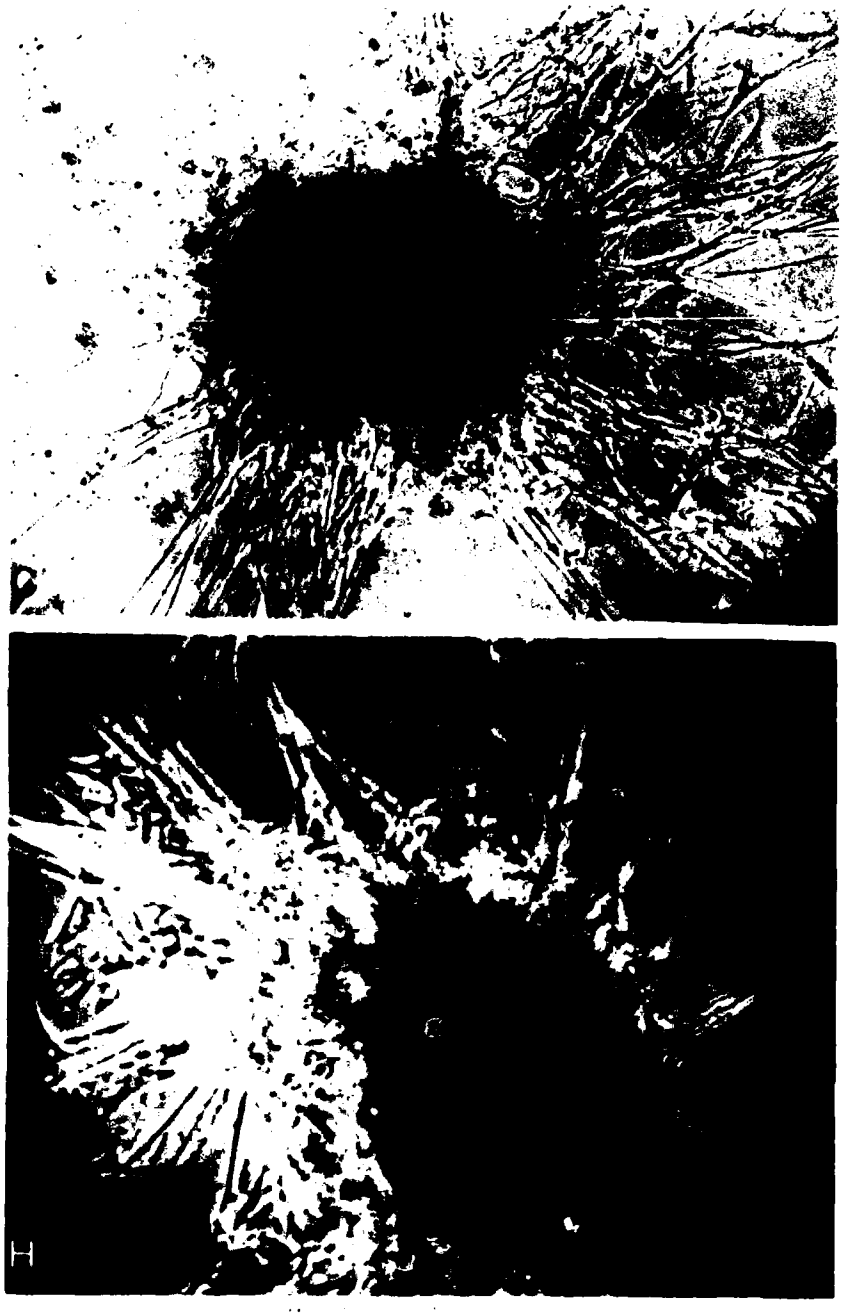
		Outer Ring		Inner Ring		
Group	Number Dishes	Number Ganglia	Mean Score ± SD	Number Ganglia	Mean Score ± SD	p
Control	9	17	3.51 ± .63	29	3.39 ± .57	ns
NGF	7	18	2.24 ± .64	12	1.23 ± .66	ns
PEMF, H	10	30	3.25 ± .92	21	3.08 ± .67	ns
DC	9	21	3.36 ± .66	26	3.47 ± .38	ns
	35	86		88		

*Significance determined by paried t test

location effects in the dish. Both 5 ml and 8 ml volumes were tested since our previous study (25) noted that growth scores were influenced by the media volume. In each case, parallel series were conducted in media containing ara C, a mitotic inhibitor, to assess growth characterisitics in the virtual absence of non-neuronal cells.

In 5 ml complete medium, or ara C-containing medium, no significant differences were obtained between control cultures and cultures treated with single pulse PEMF (Tables 1 and 2). These results indicate essentially no effect on growth parameters and are in marked contrast to those obtained in 5 ml medium with the coils oriented vertically (25). It is possible that, for these conditions, the upper limit of a dose response is exceeded by the larger current densities in the horizontal coil orientation. Analyses of the growth scores of ganglia in the inner ring, versus those in the outer ring in 5 ml complete, or ara C complete, also show no significant differences (Tables 3 and 4), indicating no correlation between neurite outgrowth and location/current density. Similar results were obtained in 5 ml PEMF experiments using vertically-oriented coils.

In cultures grown in 8 ml complete medium, mean scores of neurite outgrowth in NGF, PEMF and DC groups (Table 5) were all significantly higher than control mean scores. Although the mean score for the PEMF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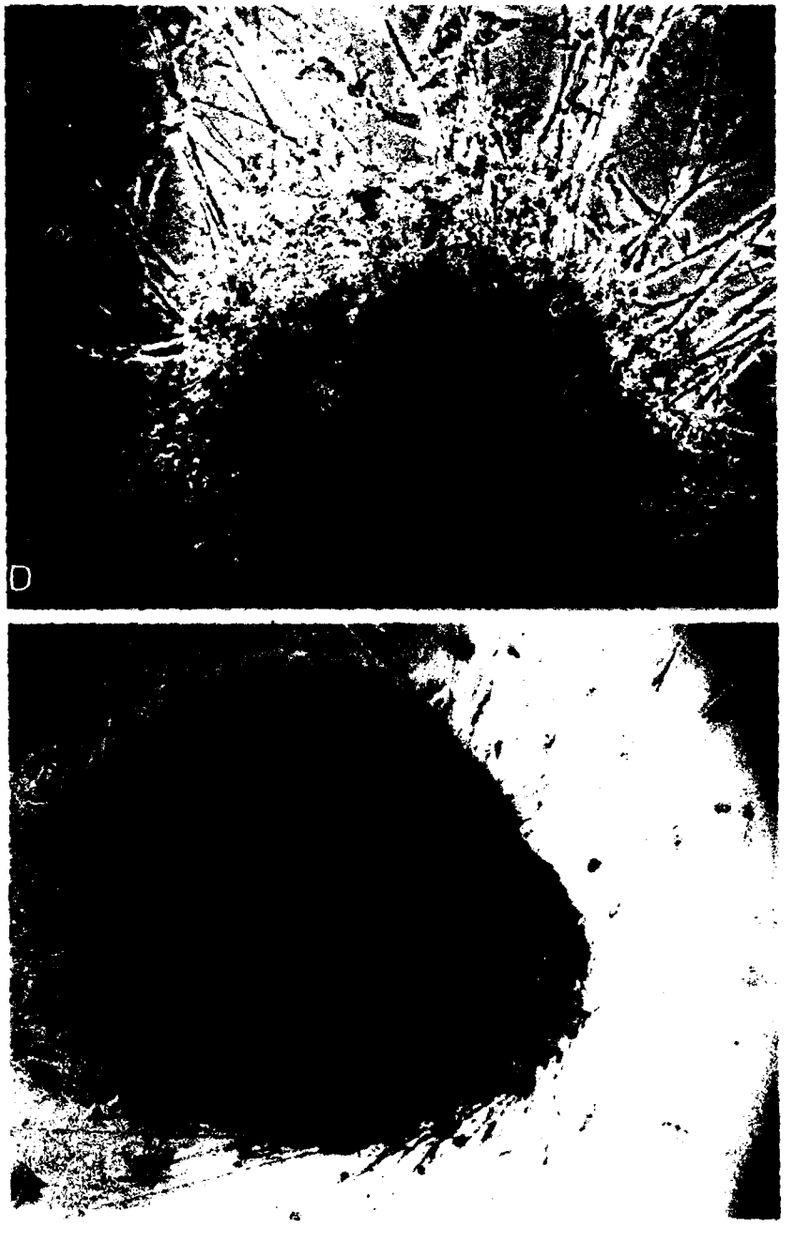
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group did not change appreciably from that found in 5 ml complete medium (equivalent distribution and level of current density), control values decreased markedly. Thus, the PEMF treatment is most pronounced when the ganglia are exposed to sub-optimal conditions , as suggested by Pilla (12,13) for PEMF effects in general. Studies confirming such phenomena have been reported by Chiabrera et al (3) and Schwartz et al (20,21).

Direct current and NGF treatments stimulated neurite outgrowth to a comparable extent as reported previously (23,24). In 8 ml complete medium, the electrical treatments proved to be beneficial to the system. In ara C medium, however, no significant differences were found in any of the treatment groups relative to controls.

Comparison of growth scores between ganglia in the same dish in 8 ml complete medium of control and NGF groups showed no correlation of growth with location, a consistent finding in all of our studies. In the PEMF group, ganglia scores in the inner ring, where the current density is low (average of 1.32 uA/cm^2), versus scores in the outer ring (average current density of 3.67 uA/cm^2) were equivalent. Closer inspection of ganglia growing in the exact center of the dish, where the current density is almost zero, exhibited growth patterns typical of those found in other parts of the dish. Between 1-3 mm from the center of the dish, the level of current density approaches that found to be most effective (0.3-0.7 uA/cm^2) in the vertically-oriented coil; however, it is most difficult to obtain such data using the horizontal system since the test intervals are too close together.

In direct current-treated cultures, the relationship between current density and growth stimulation is relatively straightforward. Although total mean scores were significantly higher in 8 cc medium, no changes in growth scores as function of location were noted. The

FIGURE 5

Dorsal root ganglia after 6 days in vitro in 8 ml medium + ara C. Pictures of whole ganglia taken under phase microscopy. Note the extensive neurite outgrowth in Control (C), PEMF (H), and DC (D), and the stunted growth in NGF (N) cultures. X80.

current density in the inner ring from 3-14 mm averaged 5.76 nA/cm^2; in the outer ring, 2.48 nA/cm^2. In 5 cc medium, a significant increase in growth scores in the inner ring (average current density of 9.1 nA/cm^2 between 3-14 mm), relative to those in the outer ring (av. 3.9 nA/cm^2), was obtained (25). In the experiments conducted in 8 cc medium, the lack of "cathodal" response of ganglia located in the inner ring appears to correlate with a lower level of current density, and may represent one end of a "current-dose" response curve. Future studies, using the same dish electrodes connected to constant current devices, will enable us to test a range of current densities from 1 nA/cm^2 to 1 uA/cm^2.

In the PEMF experiments, a correlation between growth and current density has yet to be demonstrated in the horizontal orientation. In PEMF vertical orientation, within dish stimulation of growth was correlated only with the inner ring current density of 0.7 uA/cm^2. Since this level of current density was encompassed in the PEMF horizontal experiments (at about 3.5 mm from the center), it indicates that our system may lack the sensitivity to determine growth differences in a dish where the current density is changing rapidly (0.188 uA/cm^2 per mm), and/or that other factors must be considered. For example, the interactions of the electric and magnetic components of the system have not been determined, and may play an important role in defining PEMF effects. It is intriguing to note that the total charge input to the system in the 24 hour exposure period is nearly identical (approximately 10^{-3} coulombs) for both PEMF (per polarity) and 72 hour DC experiments. This may suggest the existence of an exposure time dosage.

The mechanism of action of PEMF or DC is not well-defined (see, however, theoretical proposals by Pilla, 1974, 1980). Experiments by Cohen and his collaborators (17) and Sisken et al (24), using direct current, have implicated a reduction of calcium ion entry into the nerve cells, thus stabilizing the neurotubular and neurofilament components invclved in neurite regrowth (see Schlaepfer et al, 18,19).

In our studies, treatment with PEMF had no effect on calcium ion efflux (24). However, Bawin et al (1) described increased calcium efflux in chick cerebral tissue using 147 MHz amplitude modulated fields. More recently, Blackman et al (2) reported that odd multiples of 15 Hz sinusoidal electromagnetic fields enhanced calcium efflux from chick brain tissue. Dutta et al (4) found that 915 MHz microwave sinusoidally amplitude modulated at 16 Hz increased calcium efflux from human neuroblastoma cells. It may be that specific "windows" of applied electric field exist, whereby calcium ion changes are observed in nerve tissue. Further studies addressing this question are underway.

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IV-1

38 NERVE REGENERATION IN VITRO: CORRELATION OF CURRENT/POTENTIAL LEVELS WITH NEURITE OUTGROWTH, NEURONAL CELL NUMBER AND AREA

B.F. Sisken & E. Barr

V-1

Nerve regeneration in vitro is characterized by an increase in neurite outgrowth, maintenance of a viable neuronal population, and production of a specific neurotransmitter. It is best demonstrated in peripheral ganglia when nerve growth factor (NGF) is present in the media. NGF also acts to increase neuronal cell size. We have demonstrated the nAmp levels of direct current in vitro mimics NGF action by stimulating cathodally-oriented neurite outgrowth. Direct current levels of 10nA also appear to preserve the neuronal population as well as their specific neuro-transmitter (Substance P) content. We will now present evidence that correlates the morphological parameters of neurite outgrowth (NO) and maintenance of the neuronal population (neuronal cell areas and numbers of cells) with different current levels.

Trigeminal ganglia, obtained from 8 day old chick embryos, were placed in 60mm tissue culture dishes (Falcon 3002) containing 6ml culture media (Dulbecco MEM, 10% dialyzed fetal bovine serum, 0.6% additional dextrose, and penicillin/streptomycin). The tops of these dishes were modified to hold either platinum electrodes, tantalum electrodes, or agar salt bridges. Potentials of -200,-400,-600, and -800mV were imposed on these dishes by employing an agar salt bridge to a saturated calomel reference electrode connected to a potentiostat. Current/voltage curves were first obtained for each metal electrode and current values alone for the agar salt electrode in the tissue culture medium. In vitro experiments on the ganglia, (control cultures, cultures treated with various levels of direct current, or cultures treated with NGF, 10^{-8}M) were conducted for 3 days. Neurite outgrowth was determined semi-quantitatively in all cultures. Neuronal preservation was assessed quantitatively by counting the number of neurons in two micron sections of the explants on three levels and determining neuronal cell area on these sections with a Zeiss MOP III digitizer. Data in Fig. 1 demonstrates that neurite outgrowth is correlated with current levels rather than potential difference, i.e. maximum NO with tantalum is at -400mV(10nA) while platinum is at -200mV(12nA). Numbers of cells in ganglia treated with current (1-20nA, any electrode) and mean neuronal cell areas are all comparable. Increased levels of current decreased both parameters. NGF treatment produced higher numbers with larger cell areas. Table 1 represents a comparison of each treatment to 8 day uncultured trigeminal ganglia. Note that current levels between 1-20 nA are comparable.

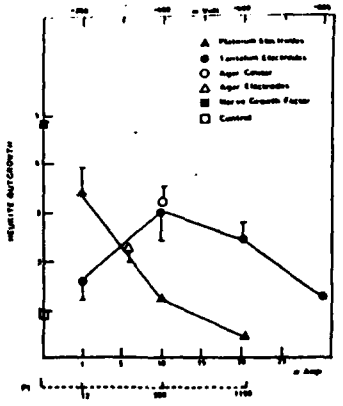

Fig. 1

Table I

Treatment		Factor
Control		1.3
1nAmp	Ta(-200mV)	1.58
10nAmp	Ta(-400mV)	1.62
20nAmp	Ta(-600mV)	1.58
10nAmp	Agar center	1.58
6nAmp	Agar only	1.86
12nAmp	Pt(-200mV)	1.58
300nAmp	Pt(-400mV)	1.05
NGF		1.97

We conclude that current levels of 1-20nA using any electrode system (platinum, tantalum, or agar salt electrodes) stimulate nerve regeneration in vitro. Supported by NSF BNS7813737 and ONR N00014-82-K-0105, Wenner Gren Research Laboratory and Department of Anatomy, University of Kentucky, Lexington, KY.

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D-11 NEURONAL REGENERATION STIMULATED BY PULSED ELECTROMAGNETIC FIELDS (PEMF) Betty F. Sisken, Bruce McLeod*, Scott Estes* and Richard Kryscio*, University of Kentucky Lexington, KY 40506 and Montana State University, Bozeman, MT 59717

Significant stimulation of neuronal regeneration after 6 days in vitro is obtained when sensory ganglia are subjected to single pulse electromagnetic fields (PEMF). Since the vertical orientation of the Helmholtz coils yields differing spatial distribution of induced fields (and hence current density), we directed our studies to determine if the degree of neurite outgrowth was correlated with the position of the ganglia in the dish. Similar experiments were performed in control dishes and in dishes treated with nerve growth factor (NGF) or direct current. The data obtained on 174 ganglia subjected to the 4 different treatments showed no statistical difference (paired t test) between ganglia located in the middle of the dish and those in the outer portions of the dish in any of the treatment groups. The Fourier spectrum of the PEMF signal was examined. It was found that the induced field pattern in space does not vary significantly with frequency up to at least 100kHz. We conclude therefore, that the major effect on the ganglia is probably due to frequency-related components of the signal at the lower end of the spectrum (less than 100kHz). This is consistent with several other recent reports. The single pulse signal may not have enough information content at the lower frequencies to produce a response that is related to position, or it may be that the neuronal cells are responding to the magnetic field component. Supported by the Office of Naval Research.

D-12 INTRAORBITAL HYPERTHERMIA WITH EXTERNAL MICROWAVES: DOSIMETRY AND THERAPEUTIC APPLICATIONS. Kenneth H. Luk*, Chung Kwang Chou, Arthur W. Guy. Department of Radiation Oncology and Department of Rehabilitation Medicine, University of Washington, Seattle, WA 98195

There are a few tumors which involve the human eye, but the impact of visual dysfunction to a patient is immense. Such examples include malignant melanoma, rhabdomyosarcoma, retinoblastoma, and metastatic cancers. Dosimetry problems and limitations in hyperthermic treatment planning include encasement of the organ by bone, fluid contents of the eye, sensitivity of the cornea and lens, and critical brain tissues behind the eye. Interstitial applications of hyperthermia can deliver the required temperature to specific regions of the eye, but it has the disadvantage of being a major anesthetic and surgical procedure. In this paper, we present experiments in phantoms constructed of pediatric and adult human skulls and tissue equivalent materials, utilizing near field microwaves. Detailed mapping is done using simultaneous recording of temperatures at multiple points by way of the computer soft-ware program of the BSD 1000 unit. The significance of this research is in the search of a practical method of hyperthermia of the eye which can be reliably reproduced and given at most major medical centers.

THE EFFECTS OF CYTOSINE ARABINOSIDE ON NERVE REGENERATION, PROTEIN CONTENT AND PROTEIN SYNTHESIS IN NGF OR ELECTRICALLY-TREATED SENSORY GANGLIA IN VITRO

B. F. Sisken[1], R. S. Estes[1], and R. Kryscio[2]

Neurite outgrowth, protein content and ^{3}H-proline incorporation into TCA-precipitable proteins were determined in 8 day chick embryo dorsal root ganglia (DRG) in four treatment groups: control, 2.5s NGF (10 nM), single pulse electromagnetic fieds (PEMF) and 10 nA direct current. All parameters were assayed for each group at 3 and 6 days in vitro (DIV). Parallel experiments and assays were conducted in the presence of 8 µg/ml of cytosine arabinoside (ara C). This drug specifically inhibits DNA synthesis and is commonly used to reduce the mitotically-active non-neuronal cells without affecting the neuronal cell population. It was used, therefore, to allow a reasonable assessment of neuronal regeneration at both the morphological and biochemical levels. Confirmatory evidence was obtained by grain localization on radioautograms after incorporation of the labeled amino acid precursor. In normal media, both electrical treatments stimulated neurite outgrowth at 3 DIV. The values were intermediate to that produced by NGF. All group values decreased by 6 DIV. Conversely, protein content increased with time due to the growth of the non-neuronal cells. In the ara C cultures at 3 DIV, neurite outgrowth increased in control cultures approximating the values of both PEMF and DC cultures; NGF cultures were unaffected at this time and remained significantly higher than any of the other groups. In drug treated cultures at 6 DIV, control, PEMF and DC neurite outgrowth increased while NGF values decreased significantly. Inspection of radioautograms of such NGF cultures revealed that the neurites grew as a circular ring within the explant rather than as the more conventional long sprouts. The protein content in these cultures diminished in all but the NGF group; these values did not change with time. Protein synthesis did not differ significantly in any of the four groups in normal media at 3 DIV and 6 DIV but diminished with time significantly after drug treatment. Radioautograms of parallel cultures contained heavy label over neurons in all groups, with densely-labeled neurites observed especially in ara C cultures, confirming that ara C did not affect the neuronal population. These data indicate that neurite outgrowth correlated closely with protein content, and DC mimics the effect of NGF.

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Soc. Neurosci Vol 9 (1983) p. 5

MORPHOLOGICAL AND BIOCHEMICAL EFFECTS OF NGF OR APPLIED ELECTRIC FIELDS IN THE PRESENCE OR ABSENCE OF NON-NEURONAL CELLS OF CHICK DORSAL ROOT GANGLIA.

B. F. Sisken, S. Estes*, E. Barr*, and R. Kryscio*. Wenner-Gren Res. Lab, Dept. of Anatomy, and Dept. of Statistics, Univ. of Kentucky, Lexington, KY 40506.

Eight day chick embryo dorsal root ganglia (DRG) were cultured in complete medium containing 10% dialyzed fetal bovine serum and 600 mg% glucose, or in complete medium with 8 ug/ml cytosine arabinoside (ara C). This drug specifically inhibits DNA synthesis and is commonly used to reduce the mitotically-active non-neuronal cells without affecting the neuronal cell population. It was used, therefore, to allow a reasonable assessment of neuronal regeneration at both the morphological and biochemical levels.

Neurite outgrowth, protein content and ^{3}H-proline incorporation into TCA-precipitable proteins were determined in four treatment groups: control, 2.5s NGF (10 nM), single pulse electromagnetic fields (PEMF) and 10nA direct current (DC). All parameters were assayed for each group at 3 and 6 days in vitro (DIV). Confirmatory evidence was obtained by grain localization on radioautograms after incorporation of the labeled amino acid precursor. In normal media, both electrical treatments stimulated neurite outgrowth at 3 DIV. The values were intermediate to that produced by NGF. All group values decreased by 6 DIV. Conversely, protein content increased with time due to the growth of the non-neuronal cells. In the ara C cultures at 3 DIV, neurite outgrowth increased in control cultures approximating the values of both PEMF and DC cultures; NGF cultues were unaffected at this time and remained significantly higher than any of the other groups.

In ara C cultures at 6 DIV, dramatic differences were noted. Neurite outgrowth in control, PEMF and DC groups increased while the NGF group decreased significantly. The protein content in the NGF cultures did not change with time.

Protein synthesis was comparable in all four groups at 3 DIV and 6 DIV, but decreased at 6 DIV in ara C medium. Radioautograms of such cultures revealed heavy label in neurons and neurite especially in the ara C series. At 6 DIV, these labeled neurites extend out radially in all groups but those treated with NGF; in these ganglia, the labeled neurites form dense circular rings around the centrally-placed neurons. Our conclusions are that ara C addition promotes neurite production by decreasing non-neuronal overgrowth demonstrating the independent ability of neurons to grow neurite processes.

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IONIC AND DIRECT CURRENT EFFECTS ON NEURITE DIFFERENTIATION IN PRIMARY CULTURES OF CHICK SENSORY GANGLIA IN VITRO

22

B. F. Sisken* and J. F. Sisken**

Differentiation of sensory neurons occurs in response to nerve growth factor or low levels of applied direct current (Sisken et al, 1982, 1983). The electric current effects may result from ionic changes at the neuronal membrane and involve changes in intracellular calcium concentrations. The purpose of this study was to: (1) examine the neurite outgrowth response of dorsal root sensory ganglia (DRG) to different levels of constant current (10 - 90 nA total current), and (2) to compare the effects of direct current (DC) to agents that modify cellular calcium levels ($CaCl_2$, $LaCl_3$, the calcium ionophore A23187 and calcium blocker, Verapamil).

Dorsal root ganglia from 8 day chick embryos were cultured in DME containing 10% dialyzed fetal bovine serum, added glucose, glutamine and antibiotics. Cultures exposed to DC had dish tops containing platinum or tantalum electrodes connected to constant current drivers. Levels of 10, 30, 60 and 90 nA total current were tested. $CaCl_2$ was added to final concentrations of 6 - 12 mM (3-4X normal); $LaCl_3$ to final concentrations of 0.1-1 mM. Calcium ionophore A23187 and channel blocker Verapamil were tested at 1 uM.

Neurite outgrowth scores were determined after 6 days in vitro, scores of sister control dishes were subtracted from those of experimental dishes. The difference in scores was expressed as a function of treatment.

Data obtained for different levels of applied constant current indicate that optimal growth occurred between 30 and 60 nA total current. Neurite outgrowth scores were clearly dependent upon calcium level modification. Those agents that increase intracellular calcium concentrations (added calcium or A23187) significantly inhibit growth to below the control level. Lanthanum chloride or Verapamil which decrease calcium entry significantly stimulate the growth response and most closely mimic the response obtained with DC.

These results are in agreement with previous reports of the deleterious effects of high levels of intraneuronal calcium and support our hypothesis that one of the modes of action of non-depolarizing levels of applied DC is to prevent calcium entry into differentiating neurons.

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May 9-14, 1982

The Correlation of Current Delivered Versus Potential Differences Imposed on Trigeminal Ganglia in vitro

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Introduction

Previous studies in our laboratory[1,2] and elsewhere[3,4], have demonstrated that low levels of direct current delivered via electrodes immersed in the tissue culture medium, stimulated neurite growth to the cathode. We also found that the neuronal population in explant and dissociated cultures of peripheral ganglia was preserved. Such phenomena represent nerve regeneration in vitro. In order to determine the electrochemical contributions in this system, we have conducted voltage/current experiments to ascertain the maximal level of this stimulation.

Materials and Methods

Trigeminal ganglia, obtained from eight day chick embryos, were placed in 60mm tissue culture dishes (Falcon 3002) containing 6ml culture media (Dulbecco MEM, 10% dialyzed fetal calf serum, 0.6% additional dextrose, and penicillin-streptomycin). The tops of these dishes were modified to hold either platinum electrodes, tantalum electrodes, or agar salt bridges. (Figure 1.) Potentials of -200, -400, -600, and -800mV were imposed on these dishes by employing an agar salt bridge to a saturated caolmel reference electrode connected to a potentiostat.[5] Current/voltage curves were first obtained for each metal electrode and current values alone for the agar salt electrodes in tissue

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Conclusions

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Neurite outgrowth was determined semi-quantitatively in all cultures. Neuronal preservation was assessed quantitatively by counting the number of neurons in 2 micron sections of the explants on three levels.

Results

Neurite outgrowth results are presented in Table 1; neuronal preservation in Table 2. For platinum electrodes, the maximal stimulation of neurite outgrowth and neuronal preservation occurred at -200mV(12nA).

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When tantalum electrodes were used, maximal neurite outgrowth occurred at a potential of -400mV(10nA), although neuronal preservation was highest at -200mV (1.5nA). When the center electrode was replaced by an agar salt bridge, significant increases in neurite outgrowth occurred at 10nA. Replacement of both electrodes, by agar salt bridges, demonstrated augmented neurite growth at current levels of 5nA. Further experiments will be done to determine current effects on sustaining neuronal cells in the agar electrode experiments.

Conclusions

Our experiments illustrate that nerve regeneration in vitro can be stimulated with direct current when current levels are between 1-12nA. The biological response of the nerve ganglia appears to be correlated with the current delivered rather than the potential imposed. Higher levels of direct current, in our system, resulted in decreased effects. Since stimulation of regeneration occurred with metal electrodes (platinum and tantalum) at low current levels as well as with agar salt electrodes, we believe that any contamination by the metal electrodes does not contribute to the stimulating effects observed.

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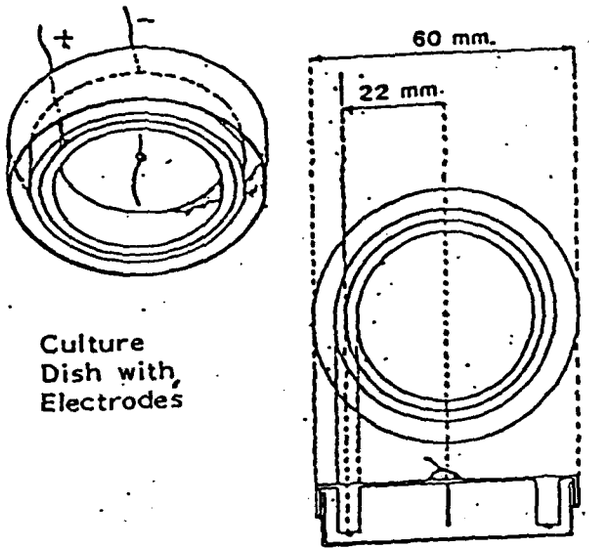

Culture Dish with Electrodes

Figure 1

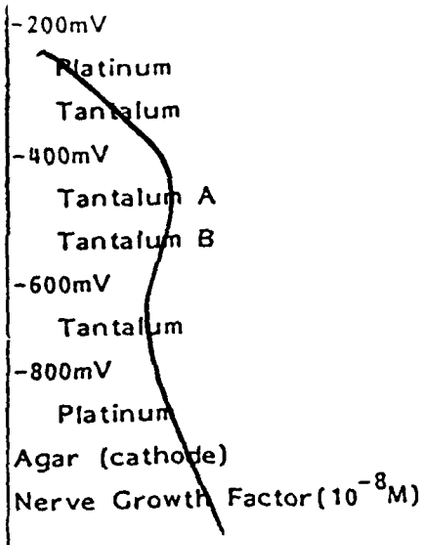

TABLE 1

NEURITE OUTGROWTH IN TRIGEMINAL GANGLIA
(0-+5 Score)

TREATMENT	NUMBER	MEAN±SEM	SIGNIFICANCE (Student's Test, Two-tailed)
Control	23	0.93 ±.18	
-200mV			
Platinum	6	3.33 ±.65	.001
Tantalum	16	1.59 ±.42	n.s.
-400mV			
Tantalum	10	3.05 ±.53	.001
-600mV			
Platinum	11	0.45 ±.20	n.s.
Tantalum	17	2.35 ±.32	.001
Agar Center			
Agar (+)	12	2.83 ±.33	.001
Agar (-)	18	3.11 ±.30	.001
Agar Electrodes Only	22	2.27 ±.24	.001
Nerve Growth Factor			
10^{-8}M	15	5	.001

TABLE 2

NUMBERS OF TRIGEMINAL NEURONS
(Total of Levels 1, 2, & 3.)

TREATMENT	NUMBER OF NEURONS
Control A	10
B	20
-200mV	
Platinum	128
Tantalum	367
-400mV	
Tantalum A	109
Tantalum B	144
-600mV	
Tantalum	60
-800mV	
Platinum	55
Agar (cathode)	52
Nerve Growth Factor(10^{-8}M)	457

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Nerve & Limb Regeneration

By BETTY F. SISKEN, Ph.D.
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The nervous system is composed of cellular units, neurons, that contact each other and their end organs (e.g., skin, muscle) through cytoplasmic processes called axons or dendrites. When the processes (fibers) between a neuronal cell body and its peripheral end organ are cut, the fibers distal to the cut degenerate completely. The fibers proximal to the cut that are still connected to the neuronal cell body degenerate for a short distance and then begin to regrow. This process of regrowth or regeneration proceeds at a rate of 1-2 mm per day. Although these nerve fibers are capable of regeneration, precise reconnections to the end organ rarely occur, and very often the end organ itself suffers through lack of "trophic (nutritive) factors" contained in the nerve fibers. Therefore, the faster the nerve regenerates, the more likely function will return. The principal goals of our current research are to understand better the process of regeneration and to use externally-applied electrical fields to accelerate this process.

The regeneration of an entire limb is obviously more complex and involves the coordinated, three-dimensional restoration of a number of tissues to replace the amputated segments. It occurs naturally in only a few vertebrate species such as salamanders and frog tadpoles. In these species, successful limb regeneration is correlated with a high ratio of nerve-to-limb surface area. Non-regeneration species such as adult frogs and mammals have a low ratio, but frogs can be induced to regenerate if the nerve supply to the limb is increased. It also has been shown that limb regeneration can be stimulated in adult frogs, and tissue regeneration in rats by applying electrical stimulation to the amputated stump. Since regenerative ability (in those animals capable of regeneration) is dependent upon an adequate nerve supply, it is possible that the mechanism by which the electric field induces regeneration is via the stimulatory effect on the nerve tissue. In this paper we will examine the effects of applied electric fields on the problems of nerve regeneration **in vitro** and to nerve and limb regeneration **in vivo**.

Modern studies on bioelectricity stem from the original experiments of Galvani (1791) who found that when an electric current was passed through a rod connecting a frog spinal cord to its corresponding muscle, the muscle contracted. The groundwork for present-day neurobiology was the discovery by DuBois-Reymond (1849) that nerves are electrically active and that they conduct a nerve impulse. However, not until the early 20th century did experimentalists apply electric currents to the problems of growth and development. The contributions of E.J. Lund from 1921 to 1947 are classics. He not only determined endogenous electric currents in plants and invertebrate animals but also applied currents to test their effect on growth. He showed that growth of onion roots, for example, could be reversibly modified by changing the magnitude, direction, and duration of the field. Both internodes and stolons of the simple multicellular animal, **Obelia**, orient to the cathode, while growth of plant sheath cells bend and grow preferentially to the anode. Orientation phenomena have been noted in other systems, as will be shown in the following sections (see also the review by Jaffe and Nucitelli, 1977).

Nerve regeneration in vitro

With the advent of tissue culture procedures, the possibility of making daily observations of nerve tissue growing in a culture dish **in vitro** under controlled conditions was made a reality. Nerve regeneration **in vitro** is defined as the regrowth of neuronal fibers (neurite out-

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growth), neuronal survival, neurotransmitter production, and restoration of contact with the normal end-organ. The first experiment to test the effects of applied electric fields on growing nerve tissue was reported by Ingvar (1920). Applying direct currents of 1-2 nA/mm^2 to chick nerve cells **in vitro**, he found that the nerve processes that grew were oriented along the lines of force. While comparable experiments by Karssen and Sager (1934) essentially substantiated these findings using current densities "less than 10 nA/mm^2," Weiss (1934), and Peterfi and Williams (1936) failed to confirm these findings, although the current densities used were considerably higher. Marsh and Beams (1946) presented a more definitive experiment by using a wedge-shaped glass chamber to grow chick central nervous tissue; the current was applied to the chamber via agar salt bridges connected to a power pack so that the current densities obtained ranged from 0.05-500 μA/mm^2. The tissue was exposed to the direct current for three to 28.5 hours. The most important observation made was that at 100-120 μA/mm^2 (10-10^5-fold higher than other studies), nerve fibers from the tissue grew preferentially to the cathode. In 1975, using a platinum electrode system and much lower current densities, we confirmed these observations: fibers from chick embryo trigeminal ganglia stimulated with direct current levels of 0.0011-11.5 nA/mm^2 were oriented to the cathode (Sisken and Smith). The rate of growth of the fibers to the cathode was 100 microns per hour. Additionally, neuronal survival in these cultured ganglia was increased from 53.5 percent in untreated ganglia to 93.1 percent in ganglia treated with direct current. The current levels used in our experiments were of the order of magnitude of those used by Ingvar and Karssen and Sager.

To address the problem of nerve regeneration **in vitro** on a long term basis, we changed our system so that larger culture dishes could be used. The configuration of the electrodes was therefore modified so that the non-uniform field used in our first experiments was maintained (see Figure 1A). Using tantalum wire for both electrodes and a 1.4 V battery, the current value after 60 minutes is 10 nA (Figure 1B; Sechaud and Sisken, 1981) The electric field obtained in this system has been calculated (Sisken et al., 1981) and is presented in Figure 2.

To determine whether the morphological effects that we have obtained are due to the current applied or the potential imposed, we employed an agar salt bridge connected to a potentiostat to set the potential in the culture dish system.

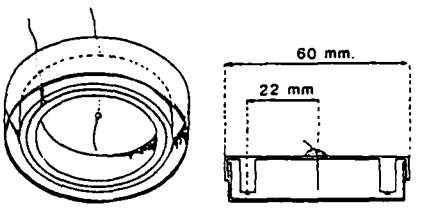

Figure 1A: Diagram of a Falcon culture dish modified to hold wire electrodes. Center cathode and circular anode are connected to a 1.4V battery.

In addition, we used: 1) dishes containing platinum electrodes instead of tantalum electrodes to test for different metal effects; 2) dishes in which the center electrode was replaced by an agar salt bridge electrode; 3) dishes with both wire electrodes replaced by agar salt bridges; and 4) dishes set up as controls (electrodes, no battery, or standards (10 nM nerve growth factor, NGF). Chick embryo sensory ganglia were placed on the bottom of the culture dish between the two electrodes. All cultures were incubated for three days at 39 degrees C. After that time, neurite outgrowth (see Figures 3 & 4) was scored, and the ganglia were fixed for subsequent histological assessment. To determine growth effects on the neurons (survival index), the areas of the neuronal cells contained with-

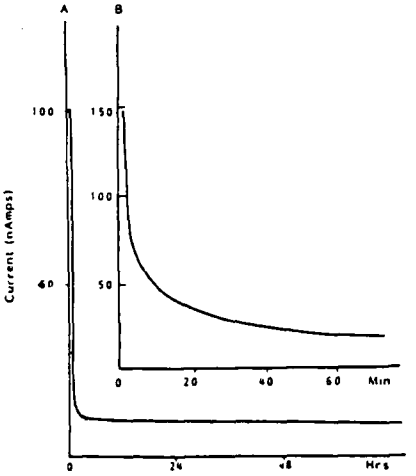

Figure 1B: Current vs. Time, current in five dishes was determined for 70 hrs (a) and 70 min (B). Examples of readings are shown in A and B.

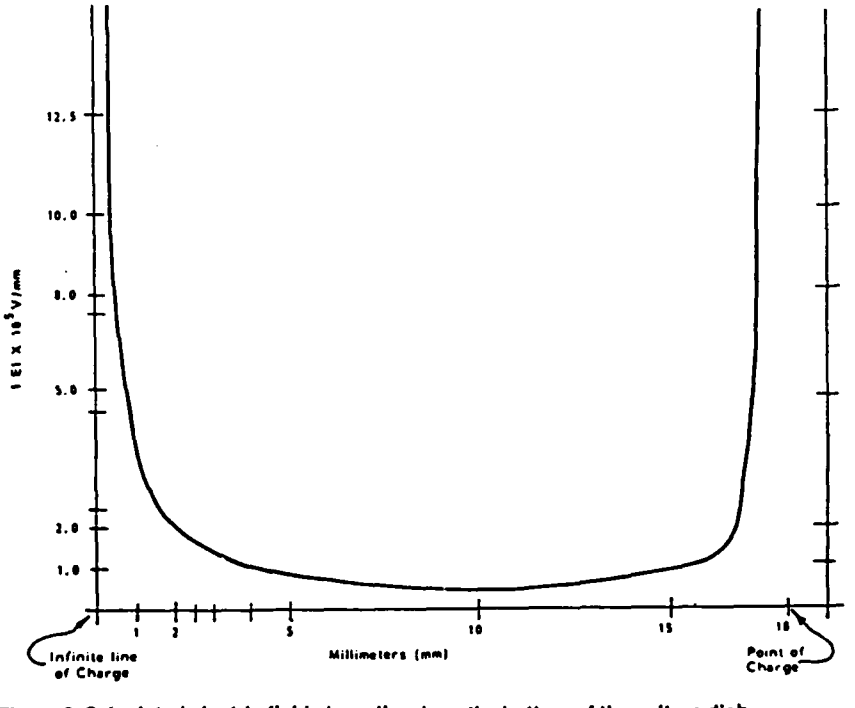

Figure 2: Calculated electric field strengths along the bottom of the culture dish.

in the ganglia were determined with a Zeiss digitizer. Both morphological growth characteristics were plotted as a function of current or voltage (see Figures 5A & 5B).

We concluded from these studies that: 1) direct current stimulation was correlated with the current applied and not the voltage imposed; 2) a "current window" was obtained in which currents between 1-20 nA are stimulatory, currents above 20 nA are ineffective or inhibitory; and 3) direct current delivered by either metal or agar electrodes produce the same degree of stimulation of neurite outgrowth, although -0.2 V applied with platinum electrodes had no effect on stimulating an increase in neuronal cell size. In all cases, standard NGF treatment stimulated nerve regeneration maximally (Sisken and Barr, 1982; Sisken et al, 1983).

We also have tested the effects of clinically-used pulsed electromagnetic fields (PEMF) on chick embryo ganglia **in vitro** and compared them to direct current or NGF treatments. Single pulse waveforms (see Figure 6A) administered for two days on a 12-hour on/off cycle stimulate neurite outgrowth at three days **in vitro**. If dissociated ganglia are cultured and treated with these fields, the numbers of neurons surviving after three days **in vitro** is increased by a factor of 1.5. Direct current-treated single neurons are also stimulated to grow and their survival rate is 1.7x greater than untreated controls (see Figure 6B).

Recently, other investigators have reported on the orienting effects of applied direct current on cultured nerve tissue. In 1979, Jaffe and Poo demonstrated that electric fields of 0.7-1.4 V/cm applied via agar salt bridges induced cathodally-directed neurites to grow faster than those facing the anode. Hinkle, et al. (1981) reported that neurites of single **Xenopus** neurons grew preferentially to the cathode in fields of 7-190 mV/mm. Also, Patel and Poo (1982) reported that fields of 0.1-10 V/cm increased the number of **Xenopus** neurons with processes in a six-hour culture. In the **in vitro** studies cited above, uniform fields of high strength were used for short time periods. Such high field strengths have been used to speed up spinal cord regeneration in the lamprey eel by Cohen and his collaborators (Roederer, et al., 1983). The low field strengths used in our laboratory (9-10 μV/mm) have been effective in enhancing partial and complete limb regeneration in frogs (Smith, 1967), and extensive tissue regeneration (regrowth of the humerus and formation of new bones) in rats (Becker, 1972; Sisken, et al., 1979 and 1982; Libbin, et al., 1979).

In summary, the results obtained from the **in vitro** experiments support the thesis that applied direct current preferentially increases neurite outgrowth to the cathode and increases neuronal survival in a manner similar to that obtained with nerve growth factor (Sisken and Smith, 1975; Sisken et al, 1983). The mechanism by which it acts may be to reduce the entry of calcium ions into the cut end of the axonal stumps, thereby diminishing the degeneration of the axonal fibers. (See Schalaepfer and Bunge, 1973; Borgens, 1981; Sisken, et al., 1981; and Roederer, et al., 1983).

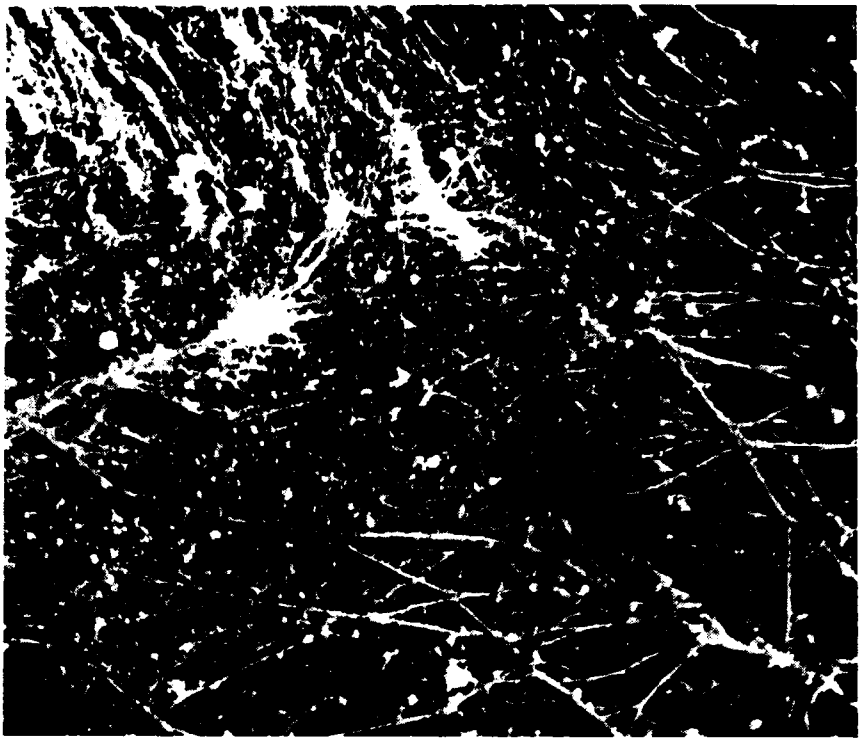

Figure 3: Scanning electron micrograph of trigeminal ganglia treated with 10nA direct current.

Nerve regeneration in vivo

The information obtained from the **in vitro** experiments has been applied to studies on nerve regeneration **in vivo**. The sciatic nerve in the adult rat is used as a model since regenerative phenomena can be ascertained within a relatively short period of time. In 1981, we reported (Winter, et al.) that the insertion of silver/platinum bimetallic electrodes into transected sciatic nerves (100 nA direct current) stimulated the rate of regeneration after 12 weeks of treatment. In this mode of application, positive results were obatined only when the silver electrode was placed distally. Mullen and Pomeranz (1982) found that 1 μA direct current (cathodal) facilitated sciatic nerve reinervation of the muscle after nerve crush, while 1 μA anodal direct current had no effect.

Preliminary work on this sciatic nerve model, using externally-applied pulsed electromagnetic fields (PEMF) in place of in-dwelling metal electrodes, appears to be a fruitful method for delivering small currents to lesioned nerves (Parker, et al., 1983). Transected sciatic nerves treated for five days with the clinical pulse burst signal regnerated faster than non-treated controls. The rate of regeneration was assayed by determining the area under the curve of the compound action potential. Similar results have been obtained in transected peroneal nerves of the cat using the same pulsed electromagnetic signal. Regeneration was evaluated by observing the numbers of labeled motor neurons in the anterior spinal cord after HRP injection into the anterior tibialis muscle of the leg (Murray, et al., 1983). Thus, the stimulatory effects noted in the **in vitro** experiments also are obtained in the living animal, using either implanted metal electrodes or pulsed elec-

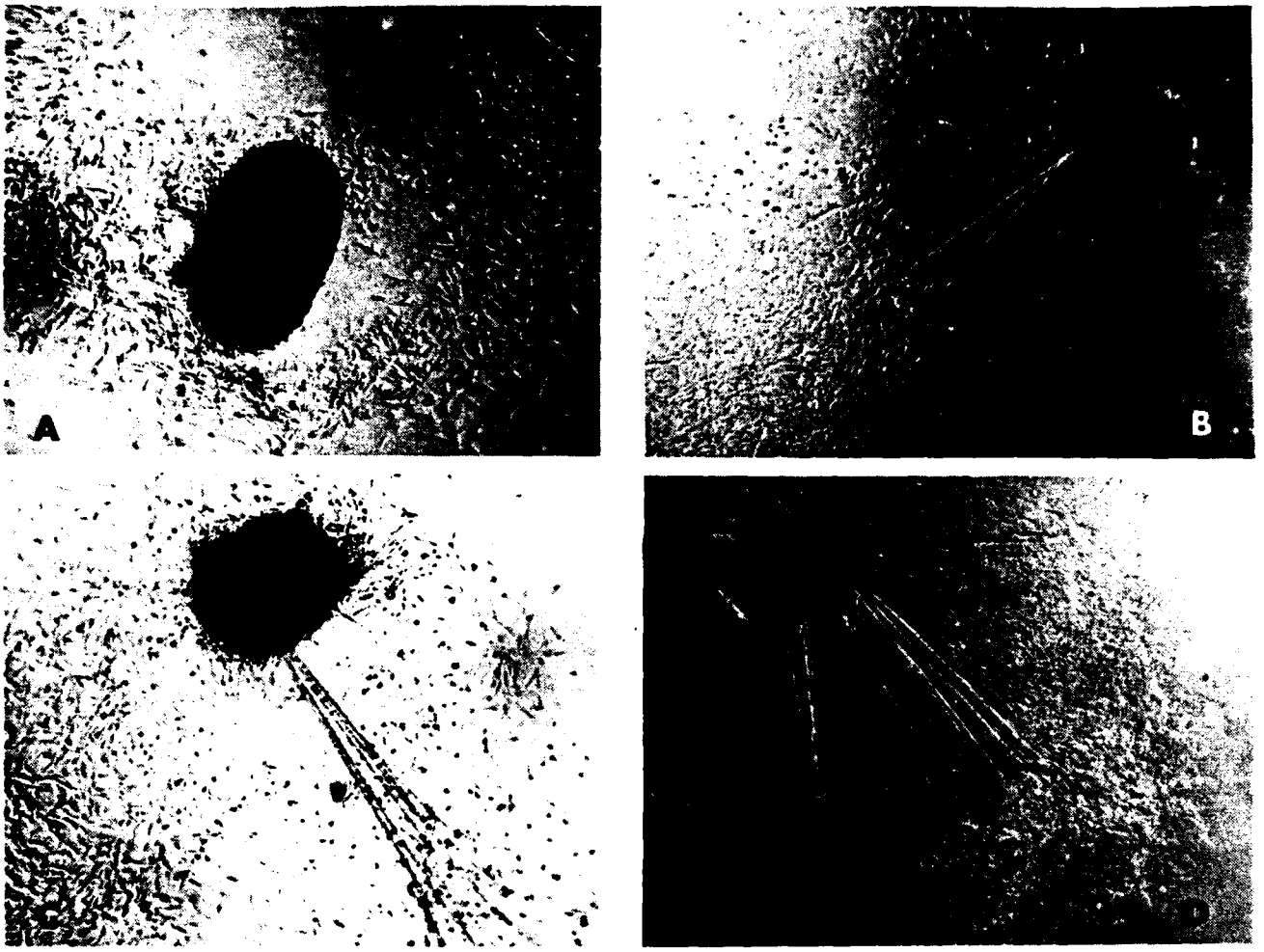

Figure 4: Neurite scoring system of sensory ganglia after three days in vitro. A, control, score 0; B, 300 nA direct current (platinum electrodes), score 2; C, 6 nA direct current (agar electrodes only), score 3; D, 10 nA direct current (tantalum electrodes), score 5. Note the orientation of the nerve fibers to the cathode located at the bottom of the photographs.

tromagnetic fields. Both types of experiments stimulate nerve fiber regrowth and both increase the survival of neuronal cells. To this end, we will continue to address both approaches in our studies.

Although the clinical use of electrical stimulation on hard tissue is commonplace, bioelectric effects on soft tissues, such as nerve, are only now being addressed seriously, and clinical application of electric fields to repair damaged nerves could be a reality in the near future.

Limb regeneration

The application of steady electric fields to the problem of limb regeneration began as a result of early investigations into determining the surface potentials of salamanders. In the early stages of salamander limb regeneration, a mass of undifferentiated cells forms under the wound epithelium and is known as the "regeneration blastema." These blastema cells are derived from the tissues of the distal third of the stump. As these cells grow and differentiate, the end of the stump elongates and eventually is restored to its former shape.

Burr (1932, 1934) measured the electric potentials on the surface of a salamander and represented the whole animal as a simple dipole with the head positive and tail negative. Monroy (1941), the first investigator to measure the potential differences along the regenerating limb of the salamander, found that the most distal regions were positive relative to proximal areas. These studies were extended by Becker (1960, 1961), who examined the changes in potential as a function of time, i.e., before and after amputation of the limb and during the regenerative phase. In these studies, he implicated the nervous system as the source of the potentials measured. To date, a direct correlation between the electric potentials measured in the limb, and the quantity of nerve tissue present, have yet to be ascertained. Recent work by Borgens, Vanable, and Jaffe (1977, 1979) designate the "Na+battery" of the skin as the source of the currents found after amputation.

Partial regeneration in a non-regenerat-

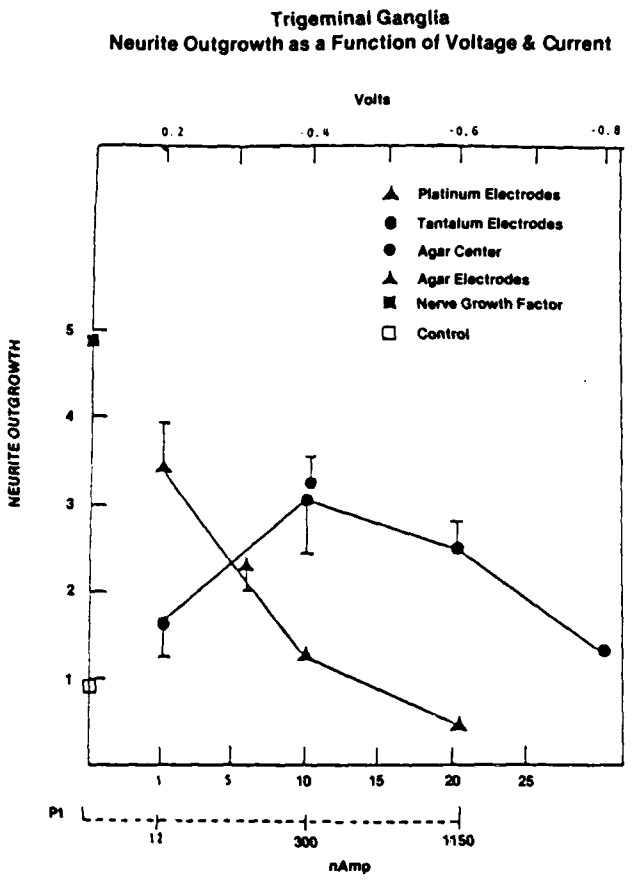

Figure 5A

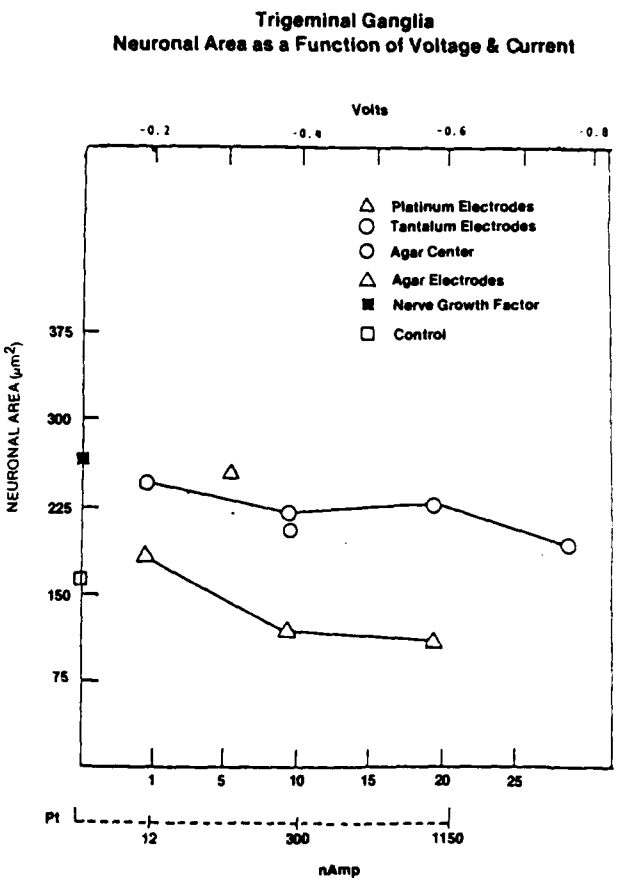

Figure 5B

ing animal (frog) was obtained by Bodemer (1964) after electrically-stimulating the nerves to the amputated stump of a frog, and by Smith (1967,1974) who applied direct current by implanting a bimetallic electrode into the stump. However, Rose (1944) had provoked this type of response by repeatedly dipping the open wound surface into salt solution, thus preventing scar formation. Implantation of electrodes into rat limbs has provoked remarkable regenerative growth (see below). No serious attempts to stimulate nerves to the stump chronically or to interfere with scar formation (except for mechanical irritation when electrodes are implanted) in a mammalian limb model have been reported. The implication from the foregoing research is that the potential for limb regeneration exists in higher vertebrates and that it can be provoked with an appropriate stimulus.

The electrical approach to the induction of limb regeneration in non-regenerating animals (adult frogs, birds, mammals) stems from the work of Becker. In 1960, he reported on the differences in "current of injury" after amputation of the limb in salamanders, a naturally-regenerating animal, and frogs, a non-regenerating animal. Whereas, salamanders exhibit a reversal of polarity from positive immediately after amputation, to negative after an interval of 10 days, the polarity in amputated limbs of frogs never reverses but slowly creeps back to the original value. He correlated the shift in polarity that occurred in salamanders with the appearance of the formation of the blastema. In frogs, the absence of the blastema and therefore the lack of polarity shift may depend upon an inadequate DC signal. Smith (1967) tested this hypothesis by implanting direct current-yielding devices (platinum/silver bimetallic electrodes) into amputated stumps of adult frongs and found that limb regeneration was stimulated. In 1974, he reported that the maximal response obtained was correlated with the insertion of the electrodes into the dorsal postaxial region of the limb. In 1977, Borgens et al repeated and confirmed Smith's experiments in the frog using a battery-powered wick electrode system. They inserted one electrode into the stump and the other electrode was left on the back. They observed that limb regeneration was induced only when the cathode was placed distally into the stump region.

In 1972, Becker modified Smith's electrode device by interposing resistors between the two electrodes and implanted this device into amputated stumps of young rat limbs. There was considerable regrowth of the distal portion of the humerus with formation of multiple epiphyseal plates only when the distal stump electrode was the cathode; unfortunately, fur-

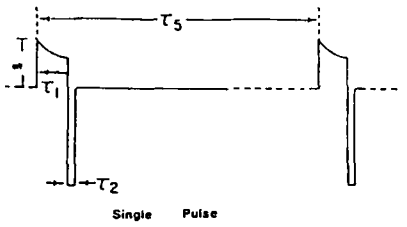

Figure 6A: Single pulse waveform used in pulsed electromagnetic field experiments. (Electro-Biology, Inc., New Jersey). T = 15mV; T_1 = 325us; T_2 = 20us; T_5 = 13.8ms, 72Hz.

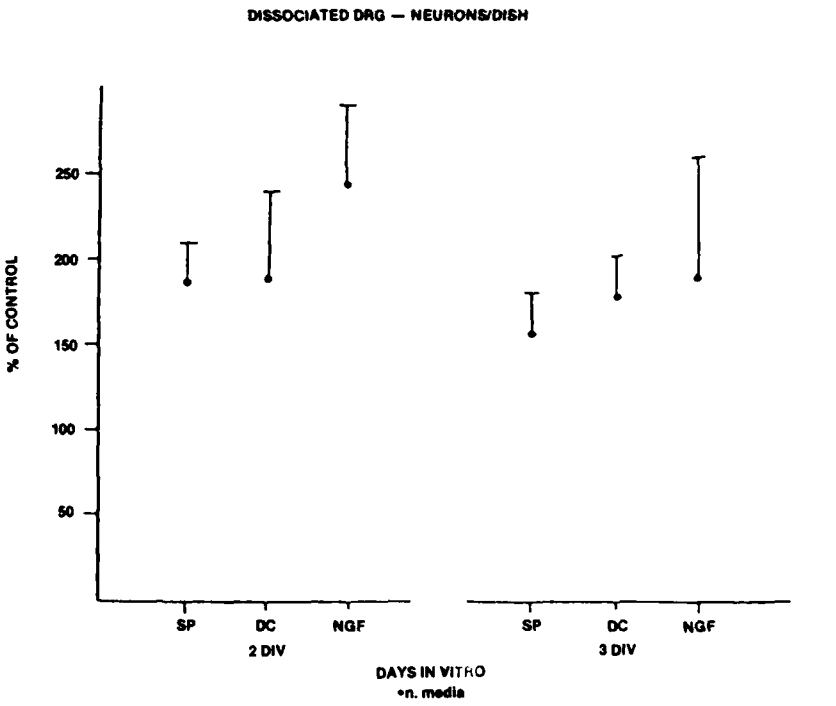

Figure 6B: Numbers of neurons after PEMF (SP), direct current (DC), or nerve growth factor (NGF) relative to controls.

ther growth of long bones from these primordia did not occur. Sisken et al.; Libben et al. repeated Becker's experiments in 1979. Our experiments involved the insertion of bimetallic electrodes alone, or in combination with nerve growth factor injections. Restoration of the humerus and formation of epiphyseal plate occurred with either treatment but the combination therapy produced the maximal increase in length of the growing humerus. Libben's experiments also showed active formation of new cartilage and bone in the vicinity of the electrode.

M. Singer and associates (1952) established the quantitative role of the nervous system in successful limb regeneration. Based on a determination of the area of the nerves in relation to the amputated limb area, they postulated that animals such as adult frogs, birds, and mammals do not regenerate naturally because they have an inadequate nerve supply. They tested this by deviating a nerve from the hindlimb to the amputated forelimb of an adult frog and found that regeneration was stimulated (1951). Application of this technique to the amputated rat limb did not induce regeneration (Bar-Maor and Gitlin, 1961).

Other attempts to augument the nerve supply to the stump include implanting fetal nerve tissue (Mizell, 1967) or embryonic neural tube (Fowler and Sisken, 1982) into the amputated stump. Presently, the implantation method appears to be the most successful.

We have developed a chick embryo model to test different techniques that may be used to stimulate regenerative processes. The amputated chick limb never regenerates, and it is a system that is easily manipulated. The implantation of bimetallic electrodes into the amputated four-day embryonic limb resulted in successful regeneration in a small number of embryos

TABLE 1

Treatment	Morphological Type			Total
	I	II	III	
Control (no implant)	38 (100%)	0	0	38
Control (plain wire)	4 (100%)	0	0	4
BME (Pt/Ag)	21 (64%)	9 (27%)	3 (9%)	33
BME — Neural Tube	9 (30%)	14 (46.7%)	7 (23.3%)	30
Neural Tube Implant	16 (27.5%)	25 (43.2%)	17 (29.3%)	58
	88	48	27	163

(see Table 1, unpublished results). The more successful technique is that developed by Mizell (1967). He inserted fetal nerve tissue into amputated stumps of newborn opossum and found that complete regeneration of the foot with digits occurred in 27 percent of his animals. We, therefore, implanted two-day embryonic neural tube into four-day embryonic limb stumps and found complete regeneration of the middle and distal segments in 29.3 percent of the cases (Fowler and Sisken, 1982). See Figure 7. The combination of nerve implant with a bimetallic electrode did not increase the percentage of successful regenerates.

Using this technique, we turned to the question of whether we could stimulate more complete limb regeneration in rats. Pieces of central nervous system obtained from fetuses of different ages were implanted into the amputated stumps of young rats. After two months, we noted X-ray positive new bones formed next to the amputated humerus (see Figure 8B). This response was obtained when the nerve im-

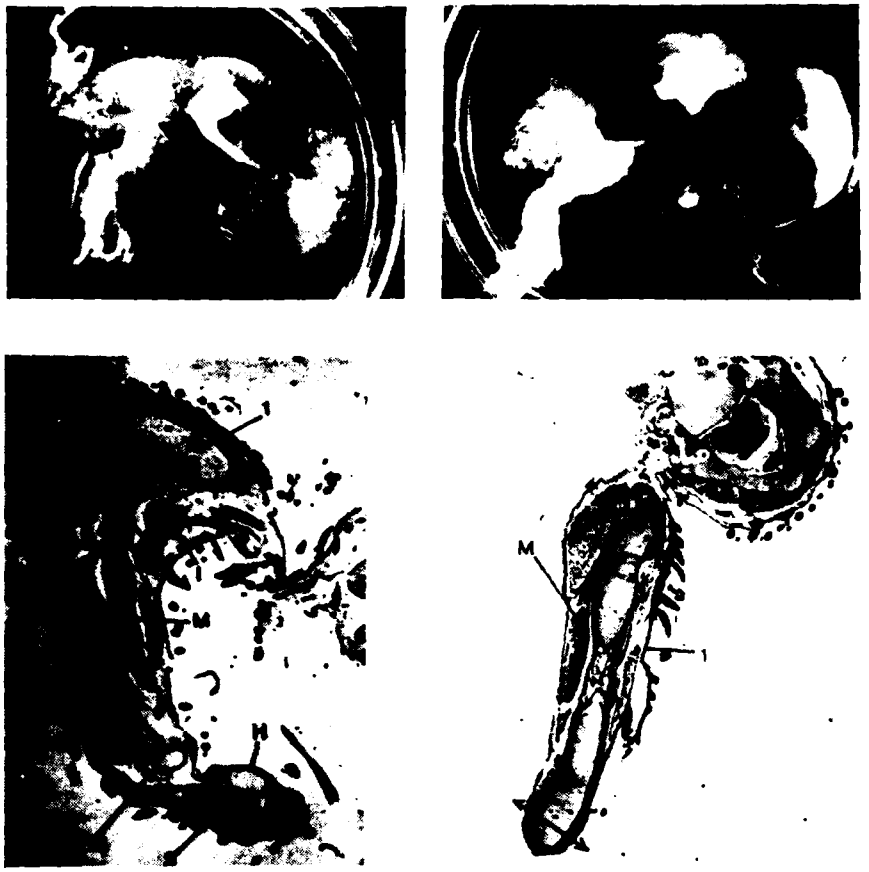

Figure 7: Photograph of chick embryos, five days post-amputation and implantation. (a) Experimental embryo that received a neural tube implant; (b) amputated limb control; (c) photomicrograph of limb shown in (a); (d) photomicrograph of limb shown in (b). (Reprinted, courtesy of Alan R. Liss Inc. (J. Exp. Zoology, 221, 1982))

plants from 12- and 14-day fetuses were used, and was never seen in unamputated limbs implanted with fetal nerve tissue (Sisken and Fowler, 1982). Most of these bones contained epiphyseal plates, and all contained healthy marrow cavities. In many instances, the new bones formed "joint-like" structures with the amputated humerus (see Figure 8D). Histological analyses of amputated limbs six months later show that these new bones have Haversian systems and contain tendinous attachments from newly-formed muscle. If this nerve implant is obtained from a 12-day fetus and inserted into the stump with a bimetallic electrode (cathode), the number of new bones formed is maximal. When nerve implants from 18-day fetuses are implanted with the direct current devices, no new bones are formed, but the longitudinal growth of the cut humerus is increased; excessive numbers of nerve fibers grow into this regenerated area (Sisken and Fowler, 1982). These results demonstrate an extraordinary amount of new tissue growth in these limbs, similar to that seen in chick embryos undergoing incomplete regeneration (designated as a Type II response, Fowler and Sisken, 1982).Although complete regeneration of the limb has yet to be obtained by any of the techniques described above, the combination therapy of applying direct current with the fetal nerve tissue implant appears to be most promising.

The mechanisms responsible for enhanced nerve fiber growth in the limb may be the direct consequence of the applied electric field on nerve growth and neuronal survival as illustrated in the in vitro and in vivo nerve regeneration experiments. The only plausible explanation for the induction of new limbs in the chick embryo and multiple new bones in the rat limb reside in the capacity of the embryonic nerve tissue to influence cells of the amputated stump. It is known that neuronal cells secrete "trophic substances" at their endings; the greater the number of viable neurons, the larger amount of trophic substances secreted. It may be that these substances are responsible for 1) acting directly on the limb tissue or 2) indirectly stimulating the host nervous tissue to induce new tissue growth.

It is too early to speculate about the clinical applications of these techniques with respect to the induction of limb regeneration since it has not been attained in the adult mammal. However, reports of the ability of children to grow back their fingertips when the amputation surface is kept open (Illingworth, 1974) suggest that the potential for regeneration may be greater than is recognized currently.

Tissue regeneration occurs in all higher vertebrates including humans. Skin, bone, nerve, blood vessels, and muscle can all be repaired to some degree. True replacement of lost parts occurs by a coordinated, three-dimensional reconstruction of the various limb tissues that eventually results in restoration of a functioning limb. This remains our goal for the future.

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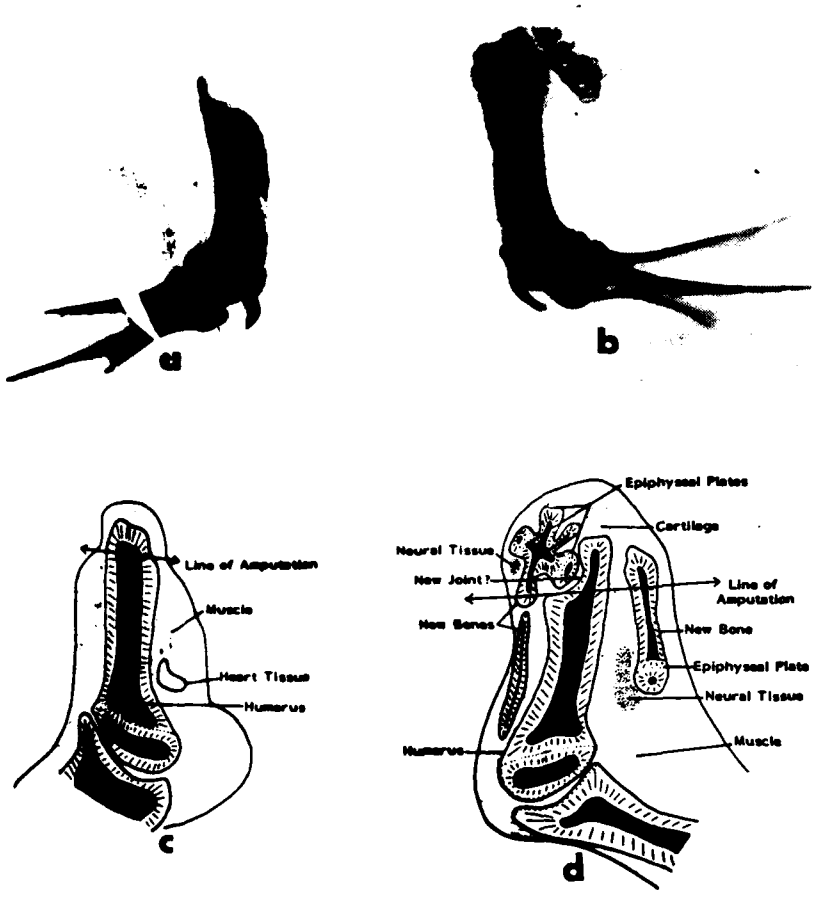

Figure 8. X-rays and diagrams of rat limbs, 2 months post-amputation. (a,c) Control limbs with implanted fetal heart tissue. (b,d) Experimental limbs with implanted fetal nerve tissue.

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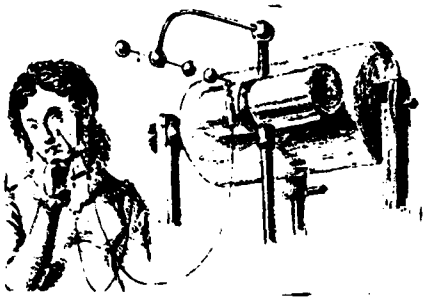

A Leyden jar provides the electrical stimulation used for application to the face to treat the pains of tic doloreux, a common ailment and treatment during the early part of the 19th century.

Illustration courtesy of Bakken Library of Electricity in Life, Minneapolis, Minnesota.

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PEMF, D.C. AND NEURONAL REGENERATION: EFFECT OF FIELD GEOMETRY

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ABSTRACT

The effects of current induced by pulsed electromagnetic fields (PEMF), or direct current (DC), were examined in cultures of dorsal root ganglia in an attempt to correlate stimulation of neurite growth with current density as a function of field geometry in the same dish. After 6 days in vitro, neurite outgrowth scores were obtained on ganglia growing in the inner ring (14 mm radius) and in the outer ring (11 mm radius) of 50 mm dishes. These scores were correlated with: ganglia growing in the vicinity of the cathode (av. current density of 28.5 nA/cm_2) in the DC group, and ganglia growing at the relatively higher current density levels (0.74 uA/cm_2) in the PEMF group. This difference in sensitivity of the neurons may be related to the method of administration of the current and not specifically to current density alone. Corollary experiments performed in the presence of cytosine arabinoside to inhibit the non-neuronal cells resulted in increased sprouting, but eliminated the differential growth in the cathode-containing ring of the DC group.

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Introduction

The effects of externally-applied electric fields on neuronal growth and function *in vitro* has been documented (Marsh and Beams,1946; Sisken and Smith, 1975; Jaffe and Poo, 1979; Sisken et al, 1981-3; Rein et al,1982). Both direct current (D.C.) and current induced by pulsed electromagnetic fields have been tested. In most cases, these studies stem directly from reports of electrical stimulation of bone and cartilage *in vitro* and *in vivo* (see volumes edited by Brighton et al,1979 and Becker, 1981; McLeod,1983).

In our laboratory, we have addressed the question of whether applied electric fields affect neuronal regeneration (neurite outgrowth and neuronal survival). We have developed an *in vitro* system for applying low levels of direct current via tantalun electrodes to peripheral ganglia (Sisken 1981), and more recently, have tested the effects of pulsed electromagnetic fields on such ganglia (Sisken and McLeod, 1982). In this report, we have examined whether the stimulatory effects of applied fields are correlated with the magnitude of the current density as a function of location of the ganglia in the same culture dish.

Materials and Methods

Dorsal root ganglia (DRG) from 7-8 chick embryos were dissected in Dulbecco's phosphate buffered saline (Gibco), and 6-8 DRG were immediately placed in 5 or 8 ml culture medium in 60 mm Falcon culture dishes. Two types of culture media were used: complete medium consisting of Dulbecco's Modified Eagle's Medium with 10% dialyzed fetal calf serum (Gibco), 600 mg% glucose, glutamine (2 mM) and 1% penicillin/streptomycin, or, complete medium containing cytosine

arabinoside (ara C, cytarabine, Upjohn) at a final concentration of 8 ug/ml. Since the addition of ara C has been used in many studies to inhibit proliferation of non-neuronal cells, a separate series of experiments in ara C-containing medium was performed to determine the effects of different levels of current density on explants consisting primarily of neuronal cells.

Each day's experiment contained four treatment groups: a control group (non-treated), a group treated with nerve growth factor (NGF) at a final concentration of 10 nM, a group treated with PEMF (single pulse, 72 Hz) for 12 hrs/day for 2 days, and a group treated for 3 days with 10 nA applied direct current (DC). Each group contained 2-4 dishes per experiment; each experiment was repeated 3-4 times.

Nerve growth factor (2.5s) was obtained from R. Bradshaw (Irvine, CA). Direct current was applied by connecting two tantalum electrodes suspended in the culture medium to a 1.4 V battery. These electrodes were configured to create a non-uniform field on the bottom of the dish; the single center electrode was the cathode, the large circular electrode was the anode. The total current measured was 10 nA. Details of this system and the current/voltage relationships have been reported (Sechaud and Sisken, 1981). The current density and electric field on the bottom of the dish has been calculated and is presented in Figure 1.

Pulsed electromagnetic fields were generated by Helmholtz coils provided by ElectroBiology Inc., NJ. The single pulse signal used in these experiments is shown in Figure 2. The dishes were placed in a column between the vertically-oriented coils; the current density in these dishes is zero at the edge of the dish, increases fairly rapidly

to a level that is dependent upon the height of the medium in the dish, and remains constant until reaching the opposite edge of the dish where it drops to zero (Figure 3). The magnetic field (5.3 Tesla/sec) is nearly constant across the dish.

All dishes were incubated in a 5%CO2, 95% air atmosphere for 6 days. In some dishes, 3H-proline at a final concentration of 10 uC/ml was added 20 hours before fixation to determine protein synthetic capability. All cultures were fixed with 3.5% glutaraldehyde in 0.1M cacodylate buffer, and scored for neurite outgrowth. The cultures exposed to 3H-proline were prepared for radioautography by fixing overnight, rinsing in 0.1M cacodylate buffer (3 changes) then in distilled water and inverting them to drain. In the darkroom, they were covered with liquid emulsion (Kodak NTB2), drained until dry, and exposed in light-proof boxes for 3 weeks at $4^{o}C$. They were developed in Dektol: water (1:2), and fixed in Kodak fix solution.

Neurite outgrowth was determined using a semi-quantitative scoring system of 0-5max based on number and distribution of neurites surrounding the centrally-placed neuronal cell bodies (Sisken et al, 1981). To determine whether there was any correlation of ganglia position with current density, a transparent guide containing concentric rings was placed under the bottom of the culture dish (Figure 4). The position of each ganglia in all dishes was noted and the outgrowth scored. The Paired t Test for Related Measures was used to determine significance of growth relative to location in the dish. Only dishes that contained ganglia in both inner and outer rings were included in these analyses. The Dunnett's Multiple-Comparison Test was used to test significant differences between the mean of all control dishes and the mean of all dishes in the various treatment

groups.

Results

Current Density and Location

At a volume of 5 ml,(height, 2.55 mm), the current density in the inner ring of the PEMF dishes (28 mm diameter)is constant with a value of 0.48 uA/cm^2 (see Figure 3). The current density in the outer ring of 11 mm radius drops off from a value of 0.48 uA/cm^2 at the boundary to zero at the edge of the dish. Most of the ganglia scored in the outer ring fell between the 17 and 24.6 mm radius location (0.48 - 0.22 uA/cm^2 current density values).

The current density in the direct current-treated dishes (Figure 1) shows that the level in the inner ring varies from an average of 28.5 nA/cm^2 (between 1- 5 mm of the cathode) to 4.4 nA/cm^2 (14 mm radius). In the outer ring, the current density is relatively constant at the level of 3.6 nA/cm^2.

Neurite Outgrowth and Location

The data obtained from these experiments is presented in Tables 1 and 2. In the complete medium series, 37 dishes containing 219 ganglia distributed in both inner and outer rings were scored. No singificant differences were found between ganglia growing in the inner ring versus those in the outer ring in control, NGF or PEMF dishes. In the dishes treated with 10 nA total direct current, ganglia growing in the inner ring near the cathode had significantly (.05) higher scores than those growing in the anode-containing outer ring (Table 1). These results confirm our previous studies demonstrating cathodal stimulation of regeneration of neurites and neuronal survival using this electrode system (Sisken and Smith, 1975; Sisken et al,

1981, 1982).

In the ara C series (Table 2), no significant differences were found in any treatment group between ganglia growing in the inner ring versus those growing in the outer ring.

Neurite Outgrowth in All Dishes

The mean value of all ganglia in each dish was determined; the values of all dishes in each treatment group were totaled and divided by the number of dishes/group to obtain a total mean score and standard deviation. A Dunnett's Multiple-Comparison Test was used to determine significance of treatment mean relative to control mean.

The data for ganglia grown in complete medium are presented in Table 3. Both the NGF and DC treatments resulted in significantly higher (.01) growth scores than the control group; PEMF stimulated growth as well but at a lower level (.05). Figure 5 illustrates the neurite outgrowth obtained in each of the groups. Note the long processes emerging from neurons that have migrated from the center of the ganglia in the DC group (ganglia located in the inner ring). Neurons in ganglia in the other groups remained in the center and the neurite outgrowth was more evenly distributed.

In ara C medium (Table 4), all values but those in the NGF group were considerably higher; significant stimulation of growth was obtained only after PEMF treatment (.05). NGF cultures were negatively affected, and direct current had no effect on the growth scores. Examples of neurite outgrowth in these cultures are presented in Figure 6. These ganglia had been exposed to 3H-proline for 20 hours before fixation. The accentuated neurite outgrowth is easily visualized in the C,PEMF and DC groups, as is the diminshed response of the ganglia treated with NGF. However, the difference in size of

the ganglia is significant; only the NGF -treated ganglia maintain a size equivalent to that found in the non-ara C culture condition.

Effect of Increasing Culture Medium Volume

Another series of experiments was run to determine if an increase in volume of culture medium (to 8 ml; height, 4 mm) and thus an increase in current density would affect the neurite outgrowth scores in the inner versus the outer rings of the culture dish.

The current density within the inner ring is Ø.745 uA/cm2, and that in the outer ring varies from Ø.745 to zero, with most ganglia growing between Ø.745 -Ø.32 uA/cm2. The data from these experiments is presented in Table 5. While no significance was found in the control dishes between ganglia in inner versus outer rings in 8 ml complete medium, a significant difference (.Ø25) was seen in those ganglia treated with PEMF growing in the inner ring at higher current density levels relative to the ganglia in the outer ring.

Neurite outgrowth total mean scores for control and PEMF groups are presented in Table 6. A t-test between two independent means was used to determine significance; in this experiment, PEMF stimulated neurite growth at the .Ø05 level.

Discussion

Our initial experiments to determine if there was a correlation between stimulation of neurite outgrowth by the application of pulsed electromagnetic fields (PEMF) with current density were inconclusive (Sisken and McLeod,1982). In this paper we have addressed this question by increasing the number of dishes in each day's experiment. In addition, we have used two different volumes of media (5 and 8 ml respectively) in an attempt to assay for different current densities

since current density is related directly to the volume of the medium when the coils are oriented vertically. Neurite outgrowth as a function of location was also determined in two other treatment groups: NGF and direct current.

Medium with and without ara C (5 cc)

The only significant difference that was correlated with position of the ganglia in the dish was obtained in cultures exposed to direct current. The higher scores obtained were found in ganglia growing in the vicinity of the cathode. Marsh and Beams (1946), Sisken and Smith (1975), Jaffe and Poo (1979) and Patel and Poo (1983) have all reported preferential neuritic growth to the cathode using cultures of embryonic nervous tissue. We have also reported increased neuronal survival in these cultures (Sisken and Smith,1975; Sisken et al, 1983). Interestingly, no significant difference was found when these cultures were incubated in ara C-containing medium.

In complete medium, the mean scores of neurite outgrowth in each of the three treated groups was significantly higher than the sister control group; NGF stimulated the greatest growth response. This differential effect has been reported previously (Sisken et al, 1981). It was observed that only PEMF provided a significant stimulatory effect in the presence of ara C. Since the non-neuronal cells have been depleted in these cultures (and some of the less differentiated neurons, Sisken et al, 1983), the total mass of the ganglia has been decreased. Protein analyses of such ganglia show that only a third of the protein (ug/ganglia) remained after incubation in ara C for 6 days. It would appear, therefore, that the remaining neuronal elements had been exposed to the electrical stimulation without any intervening cells.

Although the control, PEMF and DC cultures all exhibit extensive neurite outgrowth that exceed values found in complete medium alone, neuronal survival is decreased, demonstrating that these two processes may be independent.

Increase in volume (current density)

In this series of experiments, only two groups were compared: control cultures and cultures exposed to PEMF. The difference obtained in neurite outgrowth between ganglia growing in the inner ring and those in the outer ring was significant. The score of these ganglia (3.02) exposed to the higher current densities was comparable to the growth scores of the ganglia growing near the cathode-containing ring of the direct current cultures in 5 cc medium (2.96). A direct comparison between both types of applied electrical fields is difficult; PEMF not only has a time-varying component but a magnetic field component as well.

Although significant differences were observed with an increase in current density (by increasing the volume of the medium), an absolute correlation of growth stimulation with current density must be further tested. Presently, we are addressing this by conducting similar experiments with the Helmholtz coils oriented horizontally. In this situation, the current density is zero at the center(McLeod et al,1982) and increases linearly to a maximum at the edge of the dish.

The application of PEMF to nerve growth (Siśken et al, 1981) and to neurotransmitter release (Rein et al, 1982) in vitro has been reported. The application of these fields to the problem of nerve regeneration in vivo has been addressed more recently. Murray et al (1983)found that a clinically-used pulse burst PEMF signal

significantly increased motorneuron survival in cats after transection of the common peroneal nerve in comparison to transected untreated nerves. Parker et al (1983) reported that this same signal increased the compound action potential in transected sciatic nerves of rats relative to transected control sciatic nerves.

The mechanism of action of these externally-applied fields has yet to be elucidated. However, the effect of PEMF on ion flux changes in other systems has been documented (Johnson and Rodan, 1982; Pilla, 19). Utilization of such low current density levels eliminates the possibility of electrophoresis of surface components; more likely ion changes at the surface of the cell membrane are involved. The higher current levels (10-100 uA/cm^2) employed by Patel and Poo (1983) using uniform and non-uniform fields (direct or pulsed) appear to supply electrical energy to move membrane components. In our studies, the applied pulsed electromagnetic fields act as a trigger to stimulate growth processes. The minute currents applied cathodally by tantalum electrodes (av.28.5 nA/cm^2) to stimulate neurite outgrowth are within the range (15-30 nA/cm^2) detected by Freeman et al (1982) that flow into the growing tips of goldfish retina ganglion cells.

Acknowledgements

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TABLE 1

DORSAL ROOT GANGLIA -5CC COMPLETE MEDIUM
NEURITE OUTGROWTH RELATIVE TO LOCATION IN DISH

		OUTER RING		INNER RING		
GROUP	#DISHES	#GANGLIA	SCORE	#GANGLIA	SCORE	p
Control	11	21	2.06 ± 1.01	39	2.30 ± .49	ns
NGF	9	26	3.66 ± .65	39	3.79 ± .37	ns
PEMF	6	15	2.50 ± .63	16	1.90 ± 1.4	ns
DC	11	28	2.49 ± .9	35	2.96 ± 1.09	.05

Paired T test for Related Measures, one tailed

TABLE 2

DORAL ROOT GANGLIA - 5 CC COMPLETE MEDIUM + 8 UG/ML ARA C
NEURITE OUTGROWTH RELATIVE TO LOCATION IN THE DISH

		OUTER RING		INNER RING		
GROUP	#DISHES	#GANGLIA	SCORE	#GANGLIA	SCORE	p
Control	9	27	4.2 ± .52	22	4.07 ± .83	ns
NGF	5	18	2.37 ± .63	12	2.72 ± .44	ns
PEMF	5	20	4.73 ± .43	11	4.53 ± .36	ns
DC	6	14	3.96 ± .4	15	4.33 ± .82	ns

Paired T test for Related Measures, one tailed

TABLE 3

DORSAL ROOT GANGLIA -5CC COMPLETE MEDIUM
COMPARISON OF NEURITE OUTGROWTH BETWEEN GROUPS

GROUP	NO. DISHES	NO. GANGLIA	MEAN SCORE ± S.D.	p
Control	15	65	2.0 ± .79	
NGF	11	54	3.73 ± .58	.01
PEMF, Vertical	10	51	2.68 ± .59	.05
DC (10nA)	14	75	2.94 ± .71	.01

Dunnett's Multiple-Comparison Test

TABLE 4

DORSAL ROOT GANGLIA -5CC COMPLETE MEDIUM + 8 UG/ML ARA C
COMPARISON OF NEURITE OUTGROWTH BETWEEN GROUPS

GROUP	NO. DISHES	NO. DISHES	MEAN SCORE ± S. D.	p
Control	11	58	3.95 ± .62	
NGF	10	53	3.23 ± .75	.05
PEMF	10	61	4.63 ± .47	.05
DC (10nA)	9	47	4.51 ± .40	ns

Dunnett's Multiple-Comparison Test

TABLE 5

DORSAL ROOT GANGLIA - 8CC COMPLETE MEDIUM
NEURITE OUTGROWTH RELATIVE TO LOCATION IN DISH

		OUTER RING		INNER RING		
GROUP	#DISHES	#GANGLIA	SCORE	#GANGLIA	SCORE	p
Control	5	10	1.86 ± .89	25	2.0 ± .39	ns
PEMF	11	22	2.11 ± 1.10	45	3.02 ± 1.12	.025

Paired T Test for Related Measures, one-tailed

TABLE 6

DORSAL ROOT GANGLIA - 8CC COMPLETE MEDIUM
COMPARISON OF NEURITE OUTGROWTH BETWEEN GROUPS

GROUP	#DISHES	#GANGLIA	MEAN SCORE + S.D.	p
CONTROL	8	35	1.35 ± .87	
PEMF	14	67	2.73 ± 1.06	.005

T-test Between Two Independent Means

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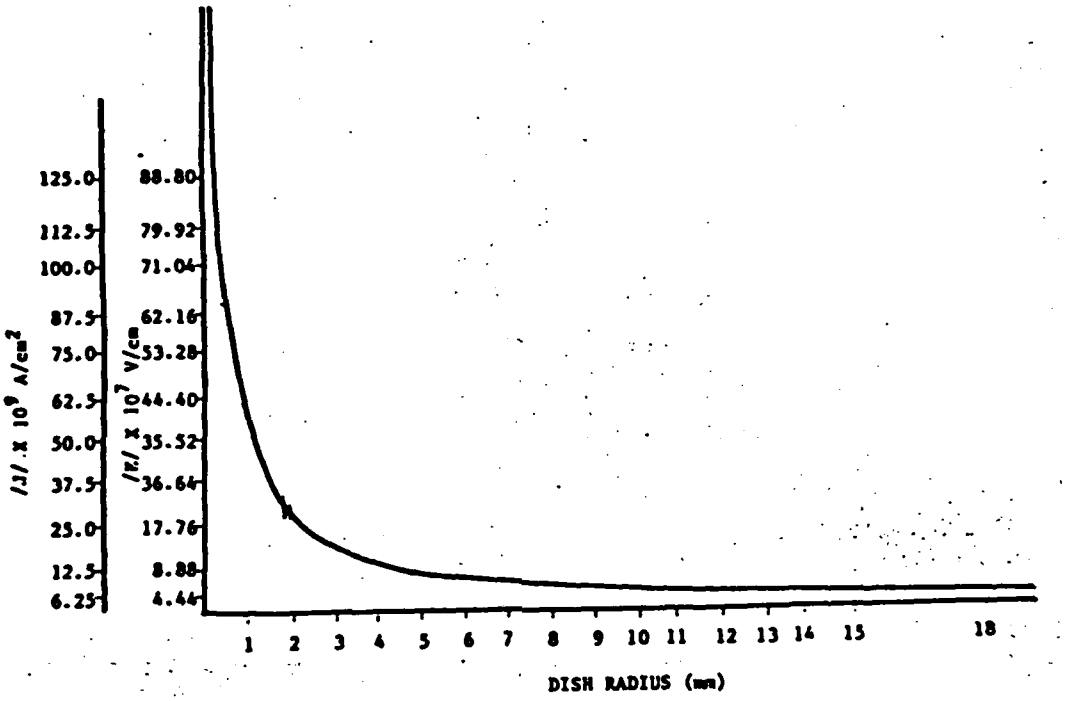


Figure 1. Electric Field and current density on bottom of dish using tantalum electrodes

$E = I \cdot R/2\ rh$
$J = E/R$
$R = 71$ ohm cmm
r = radius
h = height of medium

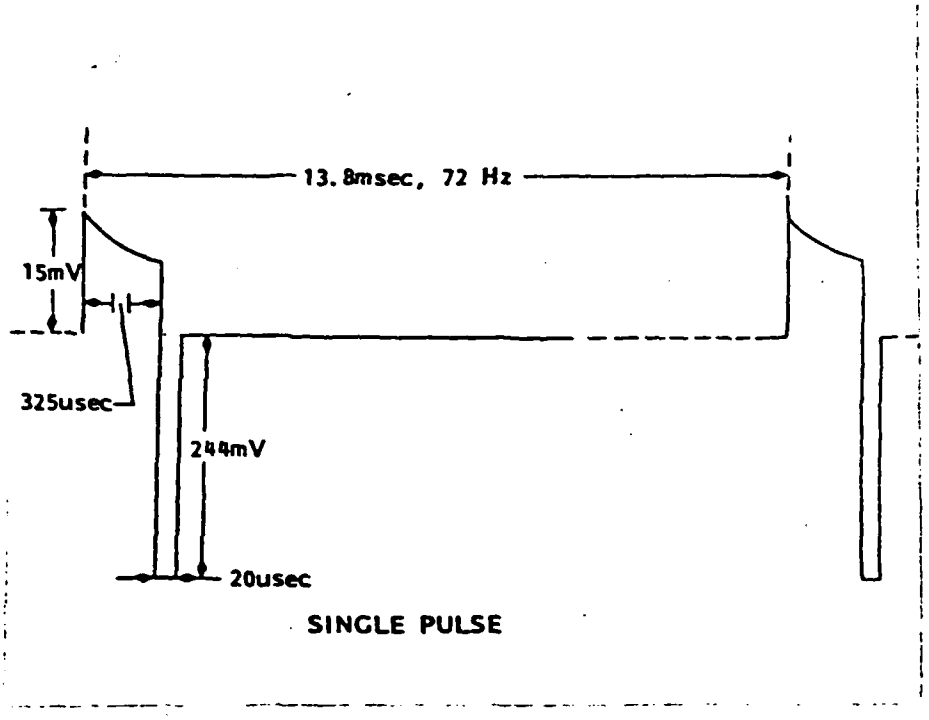


Figure 2. Single pulse waveform used in PEMF experiments.

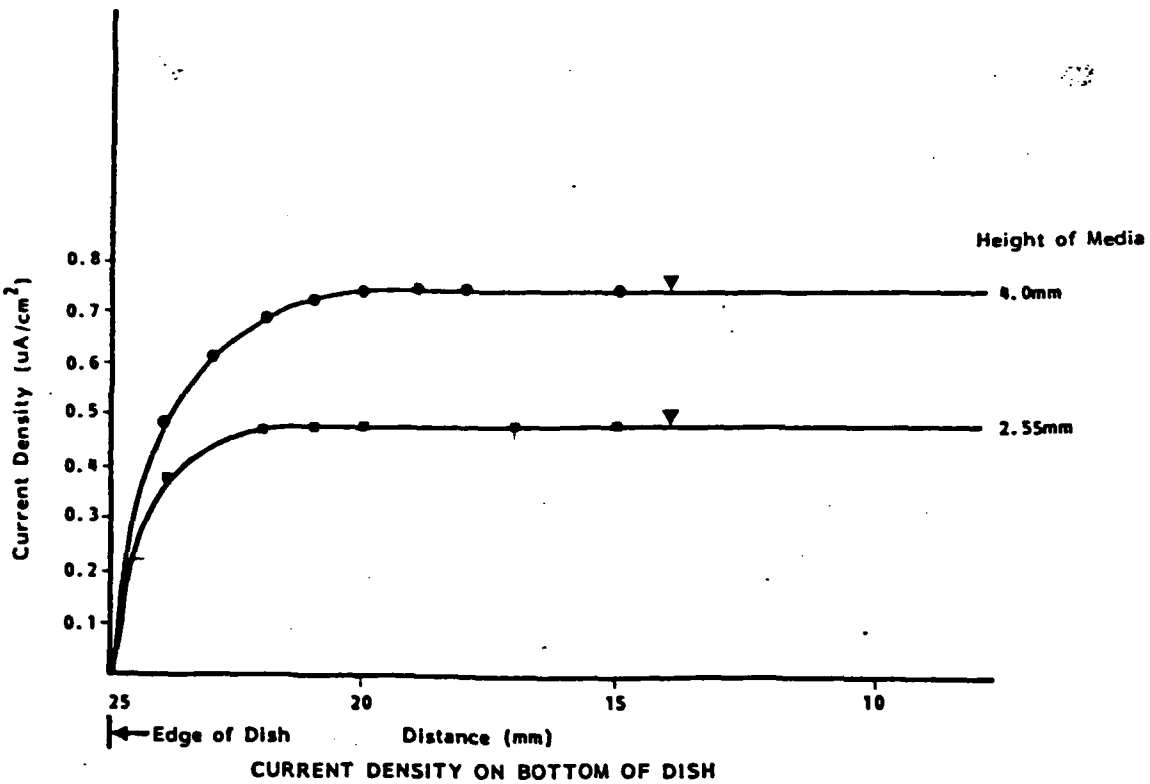


Figure 3. Current density (uA/cm^2) as a function of dish radius and height of the medium.

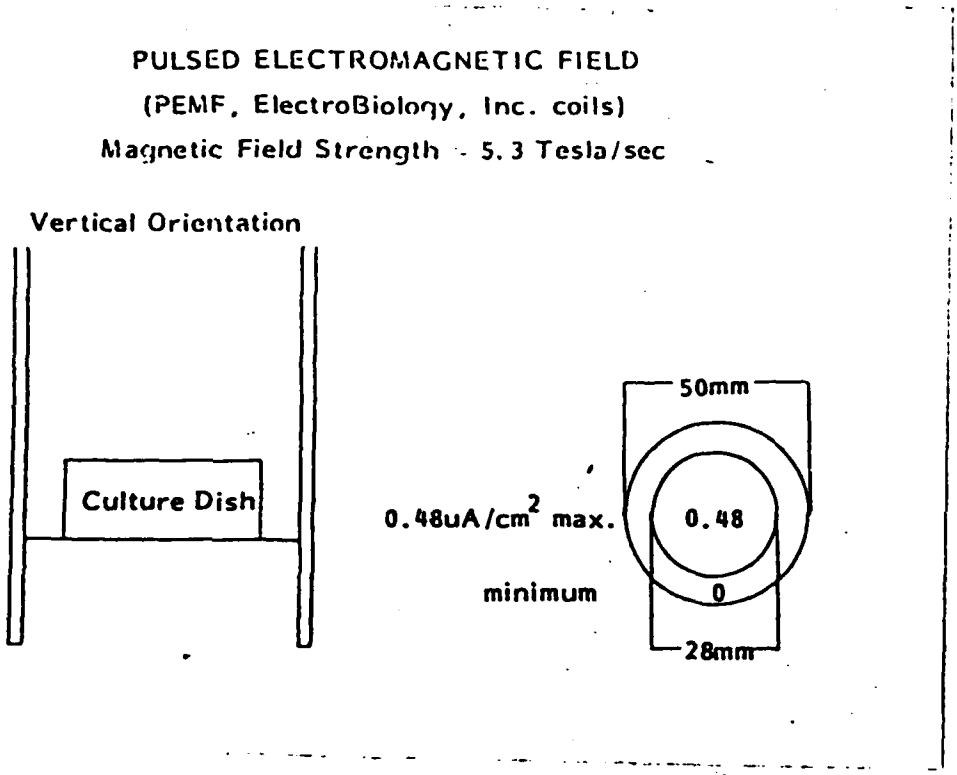
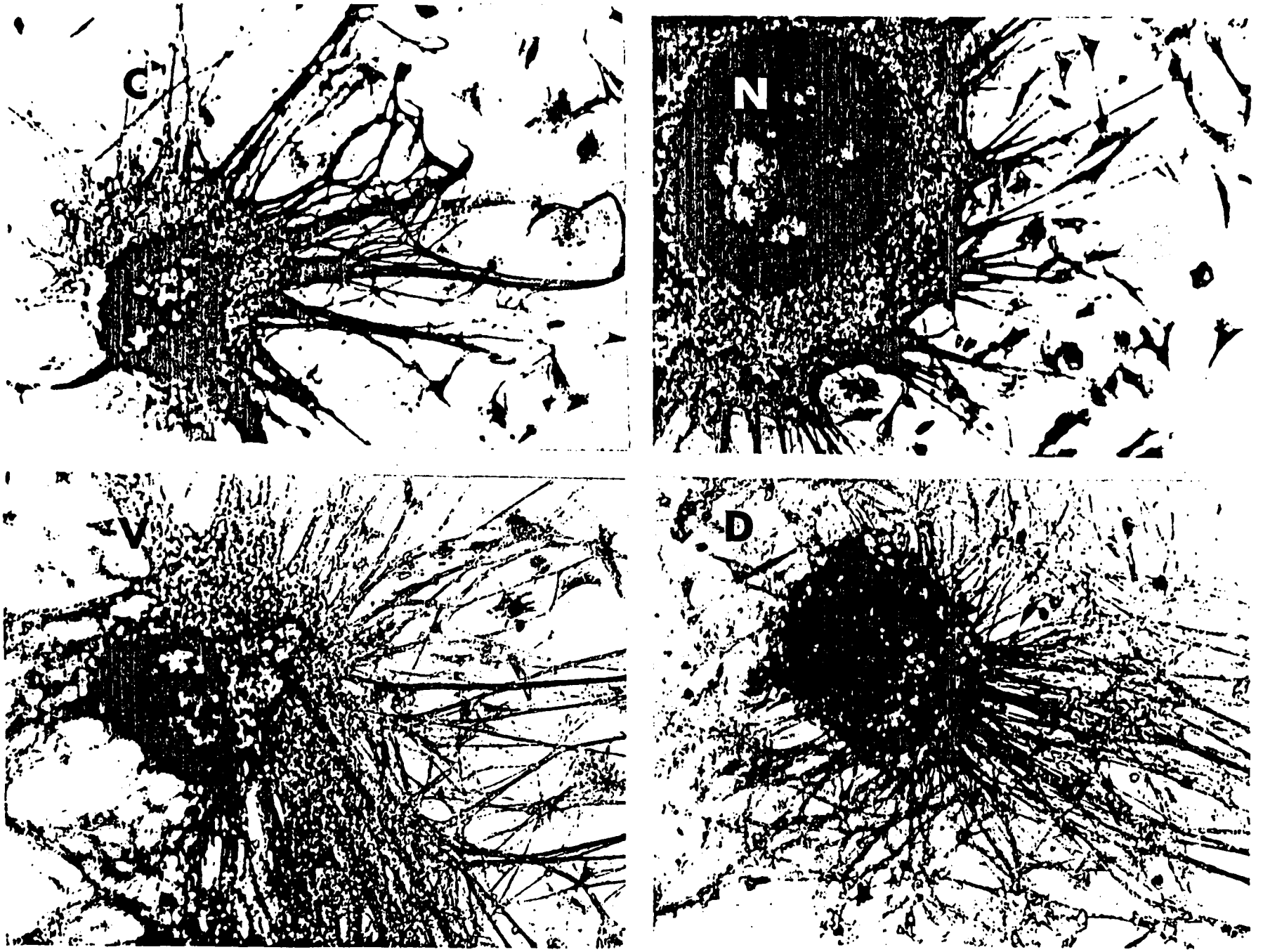


Figure 4. Placement of culture dishes between coils (vertical orientation) and current density distribution in inner and outer rings.

Figure 5. Dorsal Root ganglia after 6 DIV. Control(C), NGF(N), PEMF(V) and D.C. (D). Cajal stain. X 192. Note the neurons with long processes located at the edge of the explant in the DC group.

Figure 6. Dorsal root ganglia after 6 DIV in ara C medium. Control(C), NGF(N), PEMF(V), and D.C.(D). Radioautogaphs of whole explants after incubation in ^{3}H-proline for the last 20 hours. Neurite outgrowth in C,V and D is greater relative to that found in complete medium although the size of the explant is decresed. ~~X 192.~~ X80

A
C
D
N


C
N
V
D

Bioelectromagnetics 4:??-?? (1985)

Brief Communication

Conductivity Values of Tissue Culture Medium From 20 °C to 40 °C

A.P. Mazzoleni, B.F. Sisken, and R.L. Kahler

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Few studies are available that relate conductivity and temperature in solutions commonly used in tissue culture media. The purpose of this paper is to provide equations relating conductivity and temperature (in the range 20 °C–40 °C) for five solutions: 0.9% saline, MEM (Minimum Essential Media), horse serum, MEM with 1% horse serum, and MEM with 10% horse serum.

Key words: conductivity, culture medium, electric fields

INTRODUCTION

In vitro (tissue culture) models have been used to determine the effects of externally applied electrical fields on various types of biological systems. Such studies have employed direct current, DC [Marsh and Beams, 1946; Sisken and Smith, 1975; Poo and Robinson, 1977; Jaffe and Poo, 1979; Sisken et al, 1984, 1985], capacitively coupled fields [Brighton et al, 1984], pulsed electromagnetic fields [PEMF, Sisken et al, 1984, 1985], and oscillating [Rodan et al, 1978], and pulsing current [Aro et al, 1984]. In all cases, tissue culture media containing various concentrations of conductive salt solutions and serum concentrations of 1–10% were used.

Determinations of the magnitude of the electric field are dependent upon the conductivity of the culture medium. Few studies [Geddes and Baker, 1967] are available that report conductivity values for saline, serum, and different tissues as a function of temperature; no values are available for tissue culture media. Since several investigators are attempting to correlate biological effects with specific levels of electric fields and current densities in vitro, precise conductivity values for tissue culture media used are needed.

In this report, we present data and equations describing a functional linear relationship between conductivity and temperature for five different solutions; the equations are valid from 20 °C to 40 °C.

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The conductivities presented in this paper were determined by a four-terminal conductivity measurement [Baker and Geddes, 1971]. Such measurements require:

1) two electrodes to pass a current of known magnitude through a conductivity cell containing the solution to be tested.
2) two electrodes to measure the voltage drop for a given current.
3) calculating resistance in ohms using the relationship, R = V/I, where R = resistance (ohms), V = voltage (volts), and I = current (amps).
4) normalizing the resistance by the cell constant to obtain resistivity, and its reciprocal, conductivity.

To determine the cell constant, a solution of known conductivity was placed in the cell, the resistance was measured, the constant was calculated by using the relation $K = \sigma R$, where K = cell constant and σ = conductivity.

A four-terminal measurement that uses two electrodes to deliver current to the cell and another two electrodes to measure the voltage drop in the cell is critical in order to separate the voltage-measuring electrodes from the current-delivering electrodes. The voltage-measuring electrodes can then be placed in an area where the current density is relatively uniform. Another advantage of the four-terminal measurement is that the junction potentials between the current-delivering electrodes and the solutions do not affect the voltage measurement, as is the case in a two-terminal measurement [Baker and Geddes, 1971].

The five solutions used for this study were 0.9% saline, standard culture Minimum Essential Medium containing Hank's salts and 25 mM HEPES buffer (MEM), MEM with 1% horse serum, MEM with 10% horse serum, and horse serum alone. MEM with 1 and 10% horse serum also contained 0.6% additional glucose, 2 mM glutamine, and 1% penicillin/streptomycin; the last two solutions are comparable to those used in standard in vitro experiments. Minimum Essential Medium, serum, glutamine, and penicillin/streptomycin were all from Gibco, N.Y.

Conductivity was determined with a four-terminal conductivity cell using a 20 cc syringe (length = 0.09 m, diameter = 0.019 m) containing stainless-steel electrodes (Fig. 1A). A tissue culture incubator was used to maintain the solutions at a constant temperature which was monitored with a thermistor placed inside the conductivity cell. The conductivity determination was made by applying a sinusoidal signal of 500 Hz to the cell, using an op-amp to keep the rms value of the current constant (the magnitude of the current density used in the experiments ranged from 7 A/m^2 to 17 A/m^2). The current applied and the resulting voltage drop were then measured and used to calculate conductivity as outlined previously. An AC signal monitored with an oscilloscope was employed to avoid the electroplating of DC by-products onto the electrodes. Since an AC signal was applied to the cell, the rms values of the voltage and current were measured and used in the calculations.

Measurements were made from 50 to 1,000 Hz; no appreciable difference in conductivity was noted in this range. This implies that the conductivity is essentially resistive (ie, has a very small reactive component) and that the conductivity measured at 500 Hz is the same as would be measured with direct current. The cell constant was determined by using a standard solution of known conductivity (0.1 N KCl). The conductivity of each solution was determined at several temperatures and a linear equation relating conductivity and temperature was fit to the data for each solution

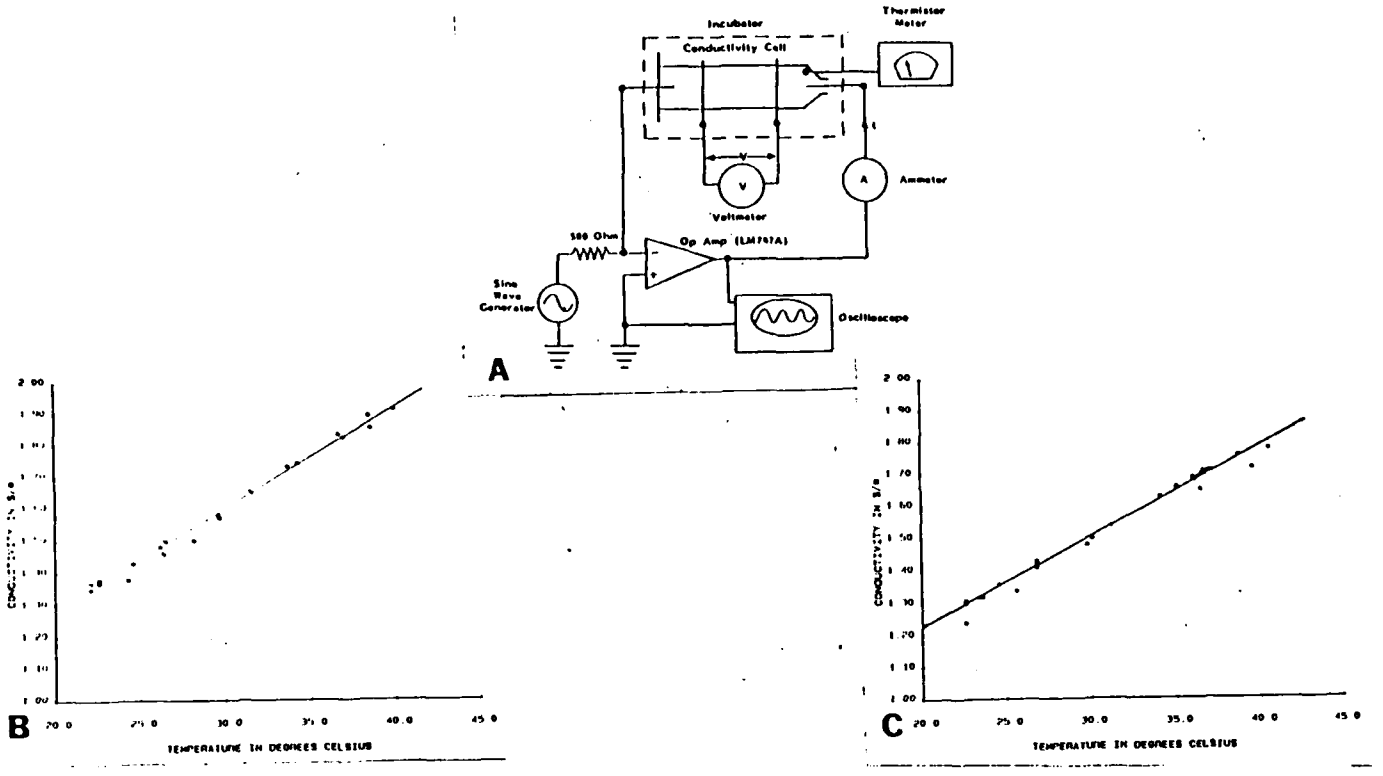

Fig. 1. Diagram of four-terminal conductivity cell (A); conductivity of Minimal Essential Medium (MEM, B); and MEM with 10% horse serum (C) as a function of temperature.

with a least-squares linear regression algorithm. Each data point represents the average of ten measurements (standard deviation = 0.005).

The measurements of conductivity versus temperature for each of the five solutions were determined; two examples are presented in Figure 1. Additionally, the equation of the best linear fit relating conductivity and temperature for each solution is represented by the continuous line on each graph. The equations relating conductivity and temperature for the five solutions are listed in Tables 1 and 2. The straight-line approximations were judged to be appropriate models in that the correlation coefficients ranged from r = .996 to r = .989. The values presented in this study are accurate to within plus/minus 5% The conductivities of the solutions at 23 °C and 37 °C are listed in Table 2.

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Of the five solutions tested, the highest conductivities in the temperature range studied were obtained with saline, 1.54 S/m at 23 °C and 2.0 S/m at 37 °C. MEM was less conductive than saline and the conductivity of MEM decreased as serum was added to it; horse serum was found to be the least conductive of the five solutions tested. The standard tissue culture medium of MEM with 10% horse serum at 37 °C was found to have a conductivity value of 1.70 S/m. The values of conductivity for saline and horse serum at 20 °C and 37 °C presented in this paper are in agreement with the values reported by Geddes and Baker [1967] for saline and cow-pig serum.

Our objective was to obtain accurate values of conductivities as a function of temperature for solutions commonly used in vitro. The values for tissue culture medium were of particular interest since it contains a complex mix of conductive salts and less-conductive serum; no systematic study of its properties has been reported. Conductivity values of two solutions, saline and serum, have been published [Geddes and Baker, 1967] and served as standard comparisons for our measuring techniques. As expected, the conductivities of all solutions tested were highest at 40 °C. Serum contains numerous species of proteins (between 5 and 8% total volume), foremost of which are albumin and various globulins, in addition to inorganic salt components.

TABLE 1. Equations Relating Conductivity (C) and Temperature (T)*

Solution	C(T)	r
Saline	.0326T + .7898 S/m	r = .996 (31 points)
MEM	.0315T + .6513 S/m	r = .996 (22 points)
MEM 1%	.0289T + .6550 S/m	r = .997 (21 points)
MEM 10%	.0284T + .6506 S/m	r = .993 (30 points)
Horse	.0263T + .6012 S/m	r = .989 (14 points)

*r = correlation coefficient.

TABLE 2. Conductivities at 23 °C and 37 °C

	S/m	
Solution	23.0 °C	37.0 °C
Saline	1.54	2.00
MEM	1.38	1.82
MEM 1%	1.32	1.72
MEM 10%	1.30	1.70
Horse serum	1.21	1.57

These large molecules, ranging in size from 40×10^3 to 20×10^6 daltons, are fairly immobile, and contribute to the decreased conductivity relative to the pure salt solution, saline.

Conductivity of all solutions declined linearly from 40 °C to 20 °C. Interestingly, the decline in conductivity with the addition of serum to the MEM was less dramatic than anticipated, and increasing the serum concentration from 1% to 10% serum had minimal effects. Electric field and current density calculations for in vitro studies can be found in the literature [Marsh and Beams, 1946; Pilla, 1974; Poo and Robinson, 1977; Jaffe, 1979; Jaffe and Poo, 1979; Sisken et al 1984, 1985]. Most calculations have used the value 1.41 S/m for tissue culture medium containing 10% serum in an experimental system at 37 °C which, in reality, is close to the conductivity of saline at 20 °C. The values obtained in this study for standard tissue culture medium containing 10% serum indicate that at 37 °C, 1.70 S/m (ρ = 58.8 Ω-cm) is the correct conductivity value. The literature contains many reports [Poo and Robinson, 1977; Jaffe and Poo, 1979; and Sisken et al, 1984, 1985] on electric field levels that have been calculated using inaccurate value of conductivity. As a result, many published effective electric field values are also inaccurate and should be adjusted accordingly.

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SOFT AND HARD TISSUE REPAIR

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11
Electrical Effects on Nerve Ganglia *In Vitro*

Betty F. Sisken, Elsie Barr and R. Scott Estes

INTRODUCTION

Electrical stimulation has been used clinically to stimulate bone healing in long term nonunions. In the early studies, metal electrodes were implanted into the fracture site and subsequent osteogenesis was observed in the region of the cathode (see Brighton, et al. 1979). This continues to be a viable approach. More recently, noninvasive methods have been developed to induce currents via applied pulsed electromagnetic fields (PEMF) to stimulate repair (see Bassett, et al. 1979). Comparable success rates are achieved clinically with either method. Basic research into the mechanism whereby applied current results in healing of the lesion has demonstrated effects on proliferation and differentiation of precursor cells.

Our studies have addressed the question of whether applied electric fields stimulate nerve regeneration. Therefore, we have developed an in vitro system using tantalum electrodes immersed in the culture medium to deliver direct current to embryonic sensory ganglia. PEMF is delivered to the ganglia by placing the culture dishes between vertically-oriented Helmholtz coils. Regenerative processes affected by the electric fields are then assayed. In vitro nerve regeneration is characterized by: (1) neuronal survival, (2) neurite outgrowth, (3) synaptic contact to the end-organ, and (4) production of the specific neurotransmitter associated with the neurons in vivo. Previous studies have shown that direct current significantly stimulated neuronal survival and neurite outgrowth to the

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cathode (Sisken and Smith 1975; Sisken, et al. 1981). Additionally, direct current is effective in stimulating neuritic outgrowth of 8-day trigeminal ganglia to 8-day co-cultured cornea; the ganglionic neurons and their processes contain positive Substance P fluorescence (Sisken and Beuerman 1983). To ascertain the mechanisms by which this stimulation occurred, we found that amino acid uptake and incorporation in direct current-treated cultures was stimulated over that of control cultures and approximated the values found in cultures treated with nerve growth factor (Sisken and Lafferty 1978).

In all cases, cultures exposed to either type of electrical stimulus are compared to sister cultures containing nerve growth factor (NGF). The effects of NGF on increasing nerve regeneration in vitro are well established (see Varon and Adler 1980), and maximal responses are obtained with concentrations of 10^{-8} to 10^{-9} M. The NGF cultures are used only as a standard for comparing effectiveness of treatment. Since NGF acts primarily as a hormone, the expectation was that the basic mechanism of action of NGF and applied electric fields might be similar but would not be necessarily identical.

In this study we have performed a series of experiments to follow the sequence of morphological and biochemical events that are associated with neuronal growth and differentiation under NGF or applied electric treatments. In addition, we have tested the effects of a commonly-used inhibitor of DNA synthesis, cytosine arabinoside, on the same properties. This drug reduces the mitotically-active, non-neuronal cell population, thereby dissociating the neuronal from the non-neuronal contribution to the regenerative process. We found that neurite outgrowth is independently regulated within the neuronal cells; it remains to be determined if neuronal survival is also independently regulated. Both of these parameters must be stimulated for regeneration to be continued. Preliminary reports of this work have been published (Sisken and Barr 1982).

MATERIALS AND METHODS

Trigeminal Ganglia

Trigeminal ganglia (TG) consisting of both ophthalmic and maxillo-mandibular lobes were dissected from 8 day chick embryos and placed in 6 ml of complete medium in 60 mm Falcon dishes (3002) with no added substrate. Complete medium consisted of 85% Dulbecco's Modified Eagles's Medium, 10% dialyzed fetal bovine serum, 3% glucose to a final concentration of 600 mg%, 1% glutamine (200 mM final) and 1% penicillin-streptomycin mixture. Each set of experiments consisted of 3 dishes/treatment; all sets of experiments were repeated 2-3 times. For each experiment, a control group (untreated) and a group treated with 2.5s nerve growth factor (NGF) 10^{-8} were run in parallel with the electrically-treated group to account for daily variations in culture conditions. All cultures were incubated for 3 days at 39°C in a 5% CO_2 incubator.

A series of experiments were conducted to test the effects of different levels of current on this system. The direct current was applied by connecting a 1.4 V mercury battery to a two-electrode system configured to maintain a non-uniform field in the dish (Figure 11.1A). The center electrode is always the cathode. Figure 11.1B shows the current vs time relationships; the details of this system have been reported (Sechaud and Sisken 1981). Either tantalum metal electrodes (0.25 mm diameter) or platinum electrodes (0.125 mm diameter) were used. Current/voltage curves for each electrode are presented in Figure 11.2 and were obtained using a three-electrode system (Figure 11.3). To discriminate between current applied and potential imposed, we used this three-electrode system to set the potential in our ganglion preparation, incubating these dishes for 3 days in the same incubator. The voltage range tested was -200 mV to -800 mV/Standard Calomel Electrode. Control cultures for this series consisted of tantalum electrodes insulated with 3 coats of plastic; no current was detected in these dishes.

To eliminate any electrode products that form as a result of direct current applied via either metal electrode, we replaced the center electrode with an agar salt bridge; the current value measured with this system was 10 nA (RCA WV511 picoameter™). Replacing both metal electrodes with agar salt bridges produced a current of 6 nA (Figure 11.4 A-B).

Dorsal Root Ganglia

Dorsal root ganglia (DRG) from 7-8 day chick embryos were dissected in Dulbecco's phosphate buffered saline and 6-8 DRGs were immediately placed in 5 ml culture medium in 60 mm culture dishes containing liners (Falcon 3006). The liners are composed of polyester (polyethylene tetrathallate) and their surfaces have been treated to be hydrophilic, thereby increasing cellular attachment. Two media were utilized in these experiments; complete medium (see above) and ara C medium consisting of complete medium with Cytarabine® (Upjohn, cytosine arabinoside) added at a final concentration of 8 µg/ml.

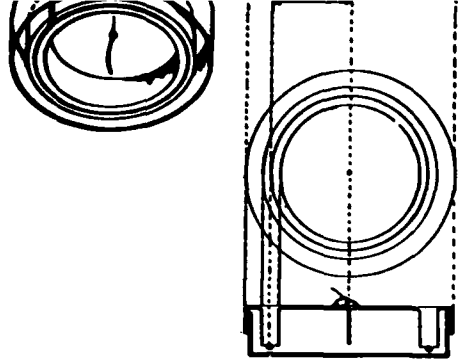


FIGURE 11.1A Diagram of a Falcon culture dish modified to hold wire electrodes. Center cathode and circular anode are connected to a 1.4 V battery.

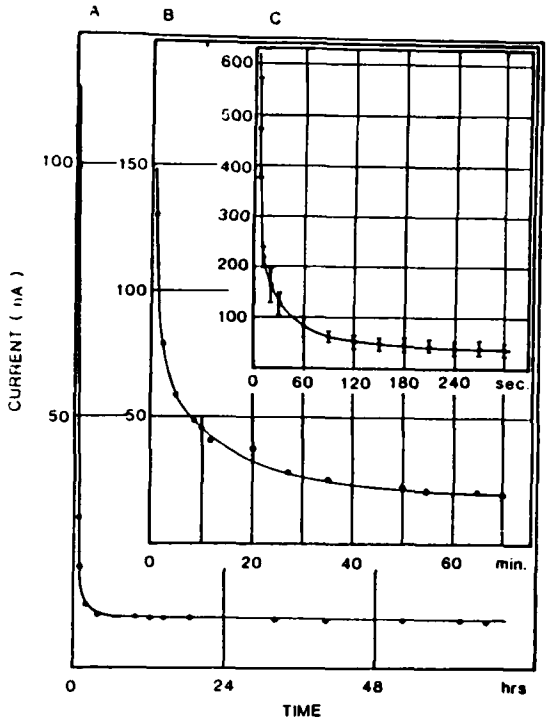
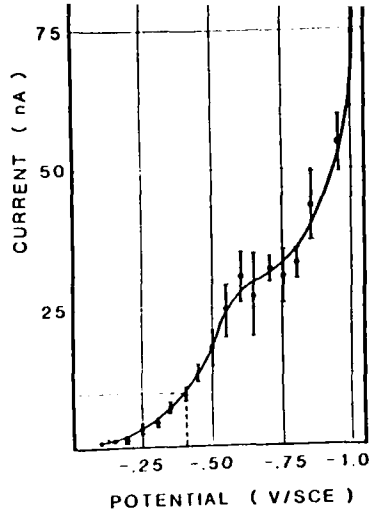
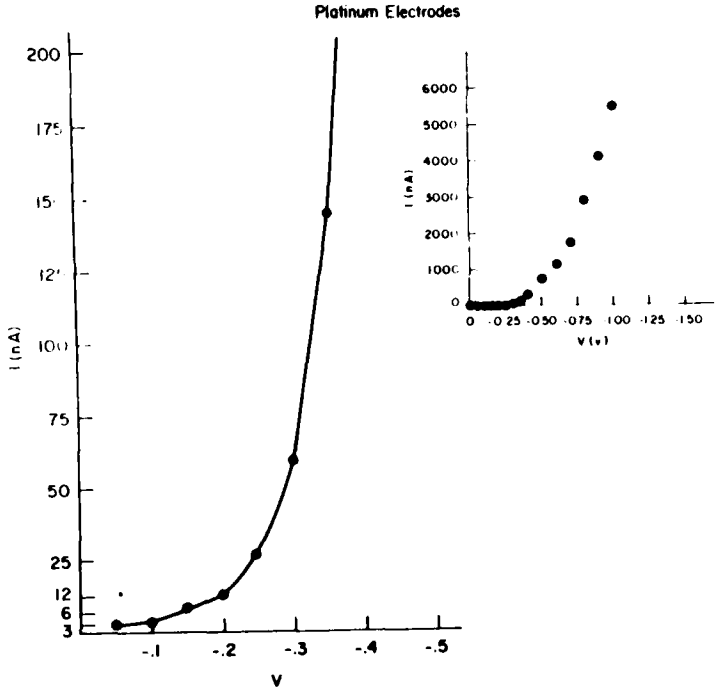


FIGURE 11.1B Current vs. time. Current in 5 dishes was determined for 70 hrs (A) 70 min (B) and 300 secs (C) in culture medium. In (C) the average of 5 dishes is shown; examples of readings are shown in (A) and (B).

FIGURE 11.2 Current vs. potential plots. Tantalum electrodes, right; platinum electrodes, below. The current was determined relative to different potentials fixed with a potentiostat (Tacussel, PRT 20-10). The reference electrode is a saturated calomel electrode separated from the dish by a salt bridge. The readings were made in culture dishes containing culture medium.





RE Reference electrode

A Picoammeter V Electrometer

SB Salt bridge

determining current vs. potential curves and for setting the potential.

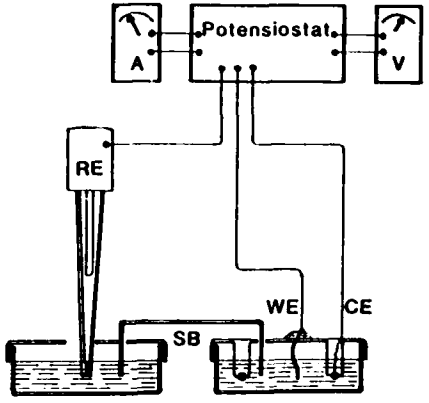
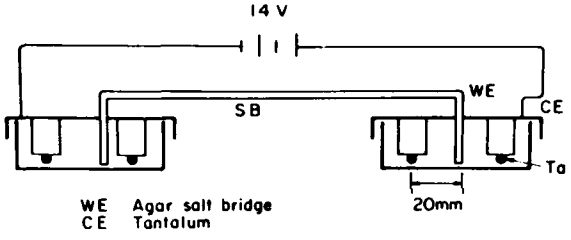
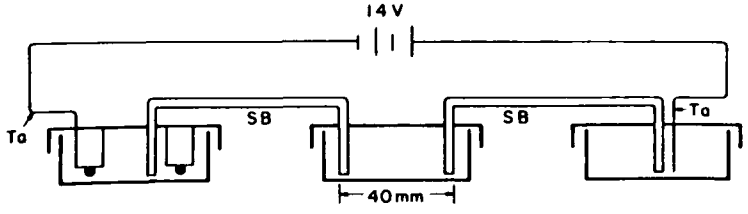


FIGURE 11.4 Agar salt bridge systems.



A. Single agar electrode (cathode)



B. Double agar electrode system

Growth Factor (NGF) group which received 2.5s NGF at a final concentration of 10^{-8} M; a group treated with pulsed electromagnetic fields (PEMF); and a group treated with 10nA direct current (DC). Each of the groups had four dishes per experiment; the duration of these experiments was either 3 days or 6 days.

The PEMF dishes were placed vertically between two Helmholtz coils and exposed for two days on a 12 hr. on/12 hr. off schedule (24 hrs total exposure). The characteristics of the PEMF waveform are shown in Figure 11.5 and it consists of a single pulse, repeated at 72 Hz. The current induced in these dishes is maximal in the center and varies non-linearly to zero at the edge (McLeod, et al. 1982). The magnetic component runs parallel to the surface of the dish and is uniform throughout.

Direct current was applied using the tantalum electrode system described above; the total current measured from these dishes was 10 nA (-400mV/SCE). The electric field created in the dish is presented in Figure 11.6.

Neurite Outgrowth

At the end of the incubation, the cultured ganglia were fixed in 3.5% glutaraldehyde in 0.1 M cacodylate buffer at room temperature for 2 hours. Neurite outgrowth was assessed on the fixed cultures using a scoring system reported in previous studies (Sisken, et al. 1981). Representative scores are demonstrated pictorially in Figure 11.7. After scoring, the cultures were refrigerated overnight.

Neuronal Survival

All cultures for these studies were rinsed twice in 4°C cacodylate buffer after glutaraldehyde fixation, post-fixed in 1% osmic acid in 0.1 M cacodylate buffer, dehydrated and processed for Epon embedding. In the TG series, propylene oxide was eliminated and pre-embedding steps consisted of equal parts of absolute alcohol and Epon. Two micron sections cut with a glass knife at 30 μm intervals, were mounted on slides and stained with toluidine blue.

Radioautography

To determine the extent and localization of protein synthesis in these cultures as a result of time and treatment, ^{3}H-proline

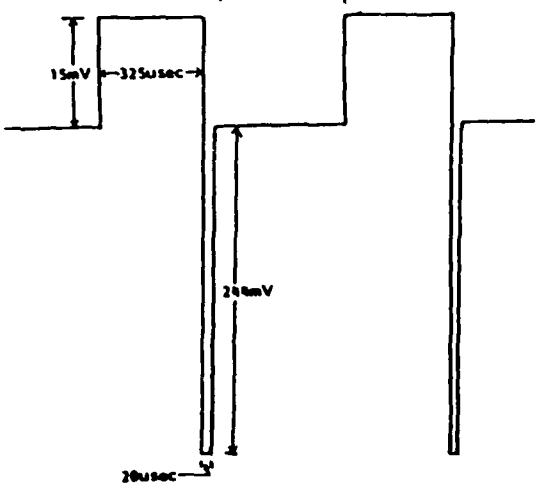
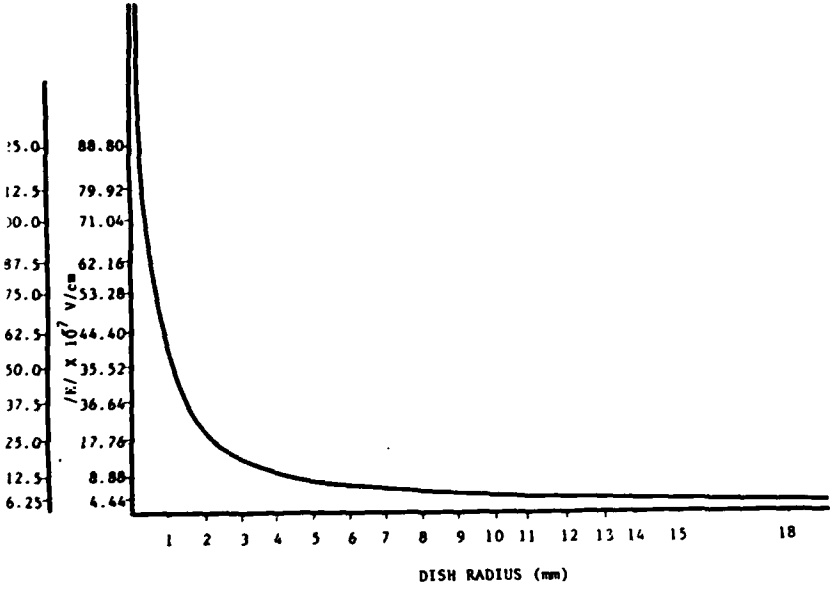


FIGURE 11.5 Single pulse waveform.
Waveform: Pulsed Electromagnetically Induced Current (PEMF)
Magnetic Field Strength: 5.3 Tesla/sec
Maximum Current Density: 0.48 uA/cm^2

FIGURE 11.6 Theoretical distribution of electric field and current density in the culture dish. See Sisken, et al. 1981 for details.



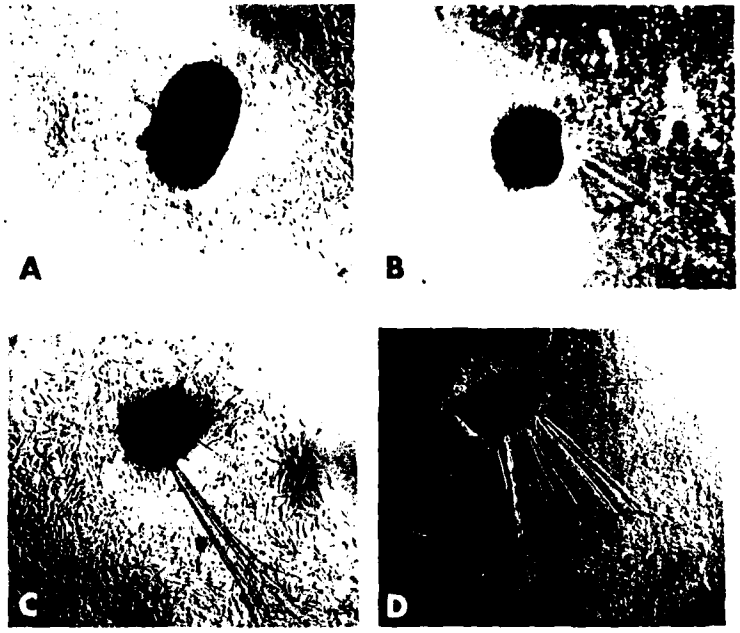


FIGURE 11.7 Neurite scoring system, trigeminal ganglia, 3 DIV. (A), control, score 0; (B) 10nA DC, score 1; (C) 6nA DC (agar electrodes only), score 3; (D) 10 nA DC, score 5. Neurites oriented to the cathode. X 50.

(L-[2,3-^{3}H-proline]), 40 µCi/mM was added 20 hours prior to fixation at a final concentration of 3 µCi/ml. After fixation with glutaradelhyde and 3 washes with 0.1 M cacodylate buffer, the culture dishes were coated with NTB2 liquid emulsion (Kodak) in the dark. The dishes were drained, inverted, and allowed to dry. They were exposed in light-tight boxes at 4°C for 3 weeks. The dishes were then developed at 18°C in Dektol, fixed, washed several times in water and dried. Photographs of the whole explant were taken with a Nikon camera attached to a Nikon inverted microscope.

All data were analyzed by R. Kryscio, Dept. of Statistics, using the approximate and exact t-test for comparing the treatment mean effects vs the control mean effects.

RESULTS

The formation of regenerated processes from embryonic sensory ganglia has been well-documented (Yamada, et al. 1971). Growth cone expansions at the end of the elongating axons contain microspikes which aid in attachment to the underlying plastic; in most cultures, these growth cones appear in the first 24 hours of incubation. Continued elongation occurs by assembly of surface material at the growth cone and uptake at the more proximal region (Bray and Bunge 1973). By 48 hours, the neurites have grown considerably, and by 72 hours have reached their maximal length. Previous studies have demonstrated that at 3 DIV, large arrays of microspikes from growth cones of trigeminal ganglia interconnect on the surface of non-neuronal cells after NGF treatment. In contrast, control cultures show limited growth cone formation. DC-treated cultures, however, have well-developed microspikes spreading over underlying cells but these appear to show few connections to microspikes from other cones (Sisken and Lafferty 1978).

Neurite outgrowth (NO) scores in trigeminal ganglia as a function of current and potential are shown graphically in Figure 11.8A. Note that at -400 mV, the total current delivered with tantalum electrodes is 10 nA. This is our standard working level for DC administration. Scores obtained at -200 mV(1 nA) and -600 mV (20 nA) are within the level of significance and fall within a dose response curve; at -800 mV the scores are not different from control values. In contrast, platinum electrodes set at a potential of -400 mV (300 nA) yield scores similar to control cultures. However, setting the potential at -200 mV (12 nA) produces NO scores that are within the range of stimulation found with tantalum electrodes. Substituting either or both wire electrodes with agar salt bridges gave current values of 10 and 6 nA respectively, and the scores overlapped with those in the "current window." In all cases, the NO scores obtained with any of the DC experiments did not reflect maximal stimulation such as that obtained with NGF, as noted in previous studies. The major findings of these experiments are: (1) neurite outgrowth is correlated with the current applied and not the potential imposed, (2) the "current window" encompasses 1-20 nA

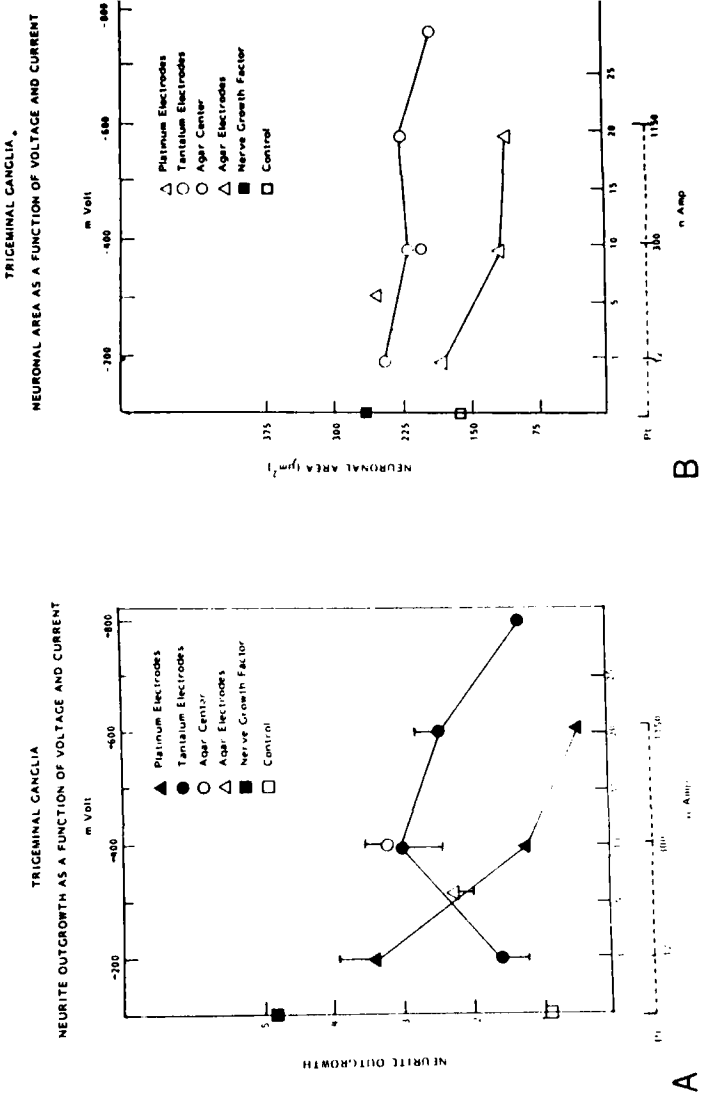


FIGURE 11.8. Neurite outgrowth (A) and neuronal area (B) as a function of current applied and voltage imposed.

do not appear to be involved negatively at these low current levels.

Neuron Survival. Two micrometer sections of ganglia fixed after any of the above treatments are being analyzed for number and area size. Data obtained thus far indicate that the same current values that stimulate neurite outgrowth cause an increase in neuronal cell area comparable to the values obtained with NGF (Figure 11.8B). Representative sections are illustrated in Figure 11.9. With increasing levels of current (20 and 30 nA), the neurons take on a more elongated appearance in contrast to the round shape normally associated with sensory neurons (Figure 9D).

In order to pursue the effects of applied electric fields on nerve regeneration at the molecular level, we turned to a more numerous population of ganglia, the dorsal root sensory ganglia located in the lumbar region. These ganglionic neurons provide sensory innervation to the hindlimbs and contain similar types of neurons as those found in the trigeminal ganglia. With the larger number of ganglia available, we were able to run simultaneous experiments adding a group treated with non-invasively applied pulsed electromagnetically-induced current (PEMF) since previous studies have demonstrated stimulatory effects on nerve regeneration. Our experimental design, therefore, expanded to study four groups; control, NGF, PEMF, and DC. Growth characteristics were assessed as a function of time and treatment in complete medium and in medium containing the DNA synthesis inhibitor, ara C. In our culture system, ara C reduces glia and fibroblasts with no obvious effects on neurite production. Future studies will define quantitatively the extent of the effect of ara C on the numbers and areas of the sensory neurons.

Neurite Outgrowth. Scores for neurite outgrowth in dorsal root ganglia (DRG) as a function of days in vitro (DIV) and treatment are presented in Table 11.1. A similar series in the presence of ara C is included. Both are shown graphically in Figure 11.10. At 3 DIV in complete medium, NO is increased with either electric treatment, and significant stimulation is obtained with NGF. All scores decreased with time and although none were significantly different from the control group, NGF, PEMF, and DC remained at a higher level. In the presence of ara C, neurite outgrowth was unaffected at 3 DIV in the treated groups, but the controls showed higher values than those

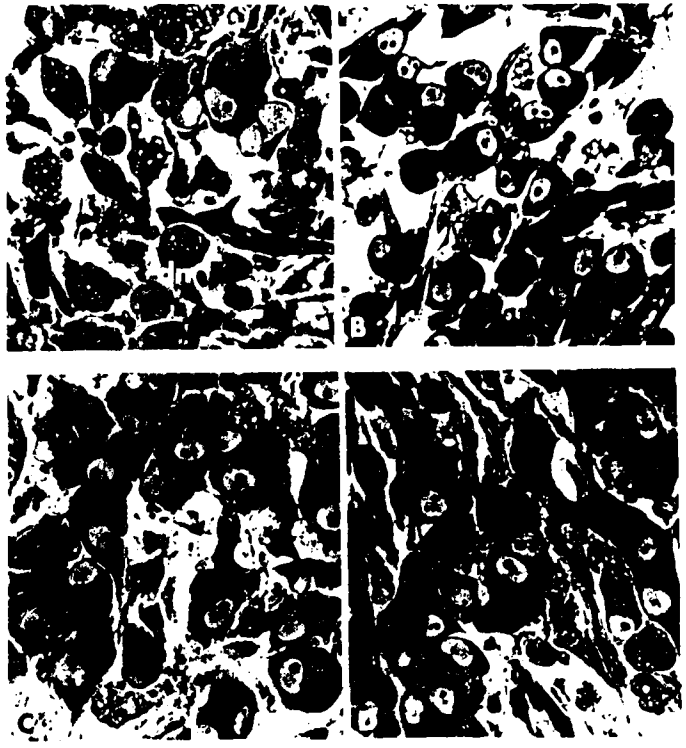


FIGURE 11.9 Two micron Epon sections of trigeminal ganglia after 3 DIV, toluidine blue stain. A, control; B, NGF, C, 10nA DC with agar salt bridge as cathode; D, 20nA DC (-600 mV) tantalum electrodes. Degenerating neurons (dn) are found in all cultures but are most obvious in control cultures. Note elongated neurons with accompanying neurites in 9 D, X 500.

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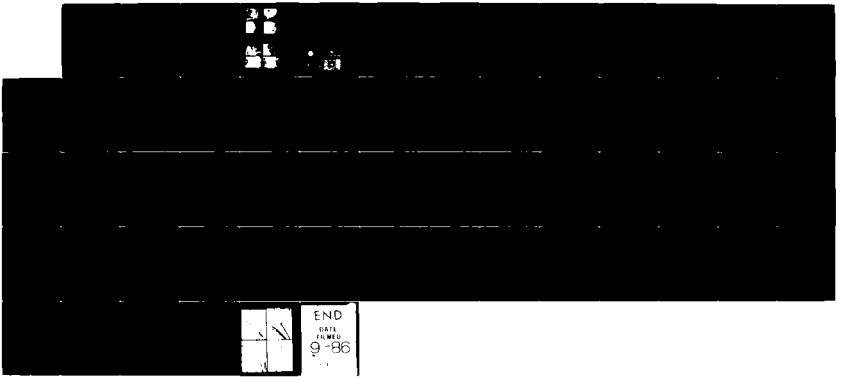

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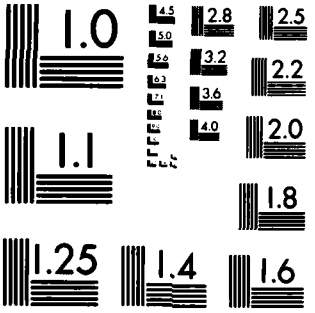

MICROCOPY RESOLUTION TEST CHART
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TABLE 11.1

Eight Day Dorsal Root Ganglia Incubated for 3 DIV or 6 DIV

	Complete Medium		Ara-C Medium	
	n Dishes	Neurite Outgrowth*	n Dishes	Neurite Outgrowth*
3 DIV				
Control	12	2.46 ± 0.17	16	3.13 ± 0.18
NGF	12	4.49 ± 0.13	16	4.32 ± 0.11
PEMF	12	3.22 ± 0.15	16	2.92 ± 0.12
D.C.	12	3.33 ± 0.25	16	2.97 ± 0.09
6 DIV				
Control	12	1.95 ± 0.23	15	4.25 ± 0.12
NGF	10	3.15 ± 0.40	14	2.55 ± 0.17
PEMF	10	2.57 ± 0.69	13	4.58 ± 0.07
D C.	13	2.78 ± 0.22	13	4.36 ± 0.10

*Mean ± S.E.M.

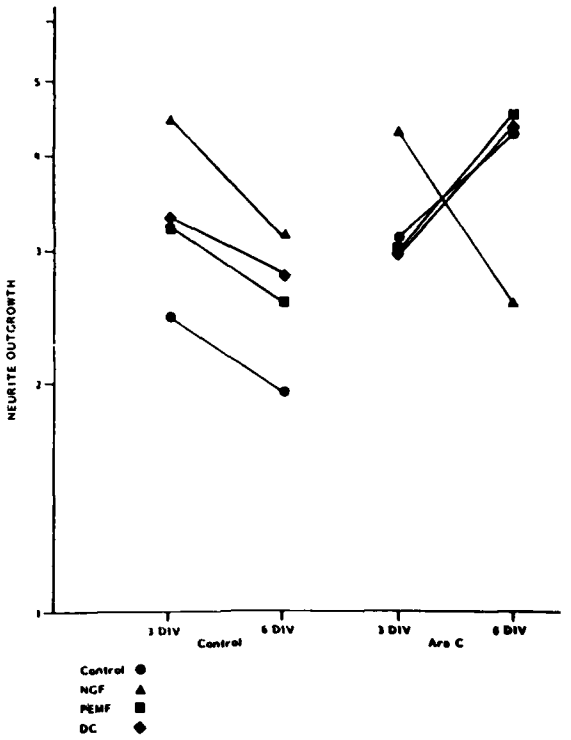

FIGURE 11.10 Neurite outgrowth in dorsal root ganglia at 3 and 6 DIV in complete medium and in medium with ara C.

obtained without the drug, so that C, PEMF and DC values overlap. At 6 DIV all scores significantly increased in these groups while that obtained for NGF significantly decreased. These effects can be seen visually under phase microscopy (Figure 11.13) or in the radioautographs of the ganglia after incubation with ^{3}H-proline.

Radioautography. Localization of protein synthesis as a result of time and treatment in the various cellular types are illustrated in Figures 11.11-11.13. In complete medium at 3 DIV, nerve cells (n) and neurites (nt) stand out as black silhouettes against the more diffusely labeled flattened non-neuronal mat (m).

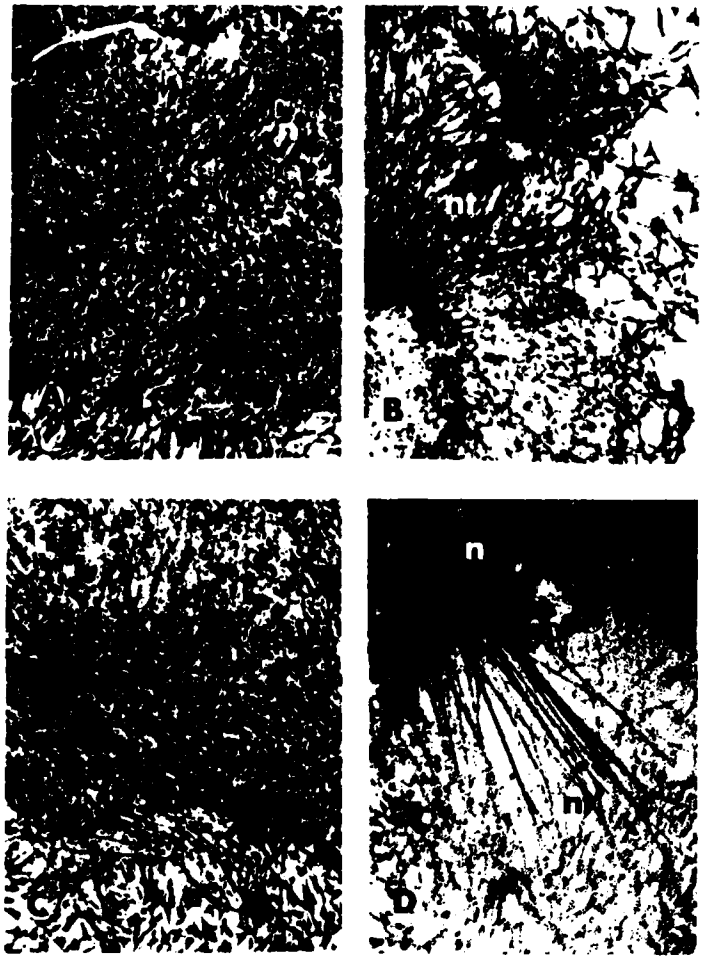

FIGURE 11.11 Radioautographs of dorsal root ganglia after 3 DIV; ^{3}H-proline added for the last 20 hrs. A, control; B, NGF; C, PEMF; D, 10 nA DC. Note black neuronal cells (n) in middle of ganglia in relief against the diffuse grains of the non-neuronal cells. Neurites (nt) contain heavy grain deposits and are prominent especially in NGF (B) and DC (D) cultures. Note black cathodally-oriented neurites in D. X 192.

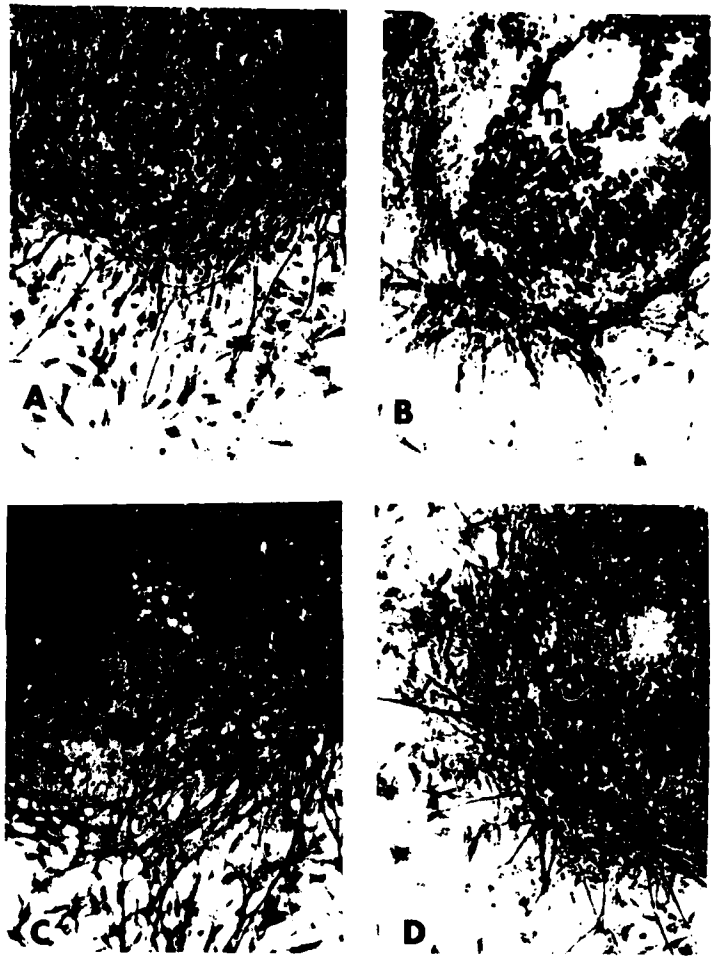

FIGURE 11.12 Radioautographs of dorsal root ganglia after 3 DIV in the presence of 8 µg/ml ara C. A, control; B, NGF; C, PEMF; D, 10nA DC. Note decreased numbers of non-neuronal cells at the periphery of the ganglia. Heavily-stained neurites are present in all four groups. Large numbers of labeled neurons are present in B and D. X 192.

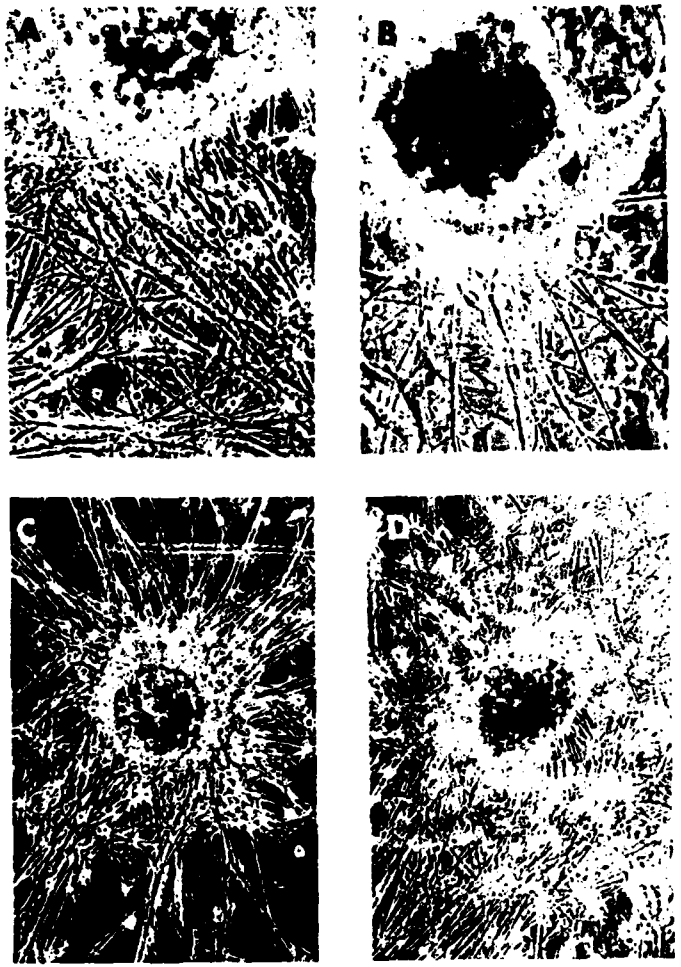

FIGURE 11.13 Phase photomicrographs of dorsal root ganglia after 6 DIV in the presence of 8 µg/ml ara C. A, control; B, NGF; C, PEMF; D, 10 nA DC. Note extensive neurite outgrowth in A, C, D. The neuronal cells in B are grouped centrally and are surrounded by circularly-arranged neurites. X 192.

Heavy label is seen especially in the NGF preparations which contain the greatest number of neurons and neurites (Figure 11.11, B). Long, heavily-labeled neurites are found in DC-treated cultures (Figure 11.11, D). By 6 DIV, non-neuronal cells have taken over and both neurons and their processes are found primarily in the center region of the explant. The neuronal population is diminished in all groups.

In ara C cultures at 3 DIV, the non-neuronal cell population has been considerably diminished and labeled neuritic processes emanate from the centrally-located neurons (Figure 11.12). Few differences between the numbers of processes and neurons are seen in these preparations. In the NGF cultures, heavily-labeled neurons are found centrally and most of the neurite processes are attenuated but still numerous. At 6 DIV (Figure 11.13), a profusion of neurites are seen extending for long distances beyond the explant in C, PEMF and DC groups; the central portion of the ganglia appears reduced in size in all groups. In NGF explants, neurites appear as relatively stubby outgrowths, but the interior of the explant is comparatively larger. Higher magnification of radioautographs of such explants reveal many neurites circling around the center of the ganglia, enclosing closely-packed neurons.

DISCUSSION

Our experiments were designed to investigate the effects of administering electric current using different modes of application to the phenomena of nerve regeneration in vitro. The first series of experiments on trigeminal ganglia assessed the effects of direct current passed through wire electrodes (tantalum or platinum) immersed directly in the culture medium. A dose response curve was obtained wherein current levels of 1-20 nA produced stimulatory effects. Current levels above 20 nA in our culture system were ineffective or deleterious. Substitution of agar salt bridges for the metal electrodes using current levels within the effective "current window" produced comparable stimulatory effects on growth. Increased neurite outgrowth and increased neuronal cell size relative to control values were obtained when direct current was applied with either metal or agar electrodes; these values approached those obtained with a standard nerve growth factor preparation. Since we are engaged in long-term studies of electrical stimulation of nerve regeneration, the metal electrode system remains the system of choice and is preferred due to its ease of handling,

minimal contamination problem and the ability to run many cultures concurrently.

We have expanded our studies on the trigeminal ganglia by looking at trophic interactions between the ganglia and co-cultured cornea in the 8 day chick embryo using direct current or NGF to increase outgrowth to this end-organ. Pre-incubation in cyclohexamide (10 µg/ml) was necessary to inhibit corneal epithelial overgrowth leaving the neurite processes unaffected. Synaptic-type contact between trigeminal neurite endings and the corneal stroma were obtained. The neurotransmitter (Substance P) was localized in neurons and neurites in such cultures (Sisken and Beuerman 1983).

The data obtained on cultures of dorsal root ganglia after stimulation with either direct current or pulsed electromagnetically-induced current show less difference relative to control cultures than that obtained in the trigeminal cultures. This may be due to the special liners, but more probably involves the higher growth potential of these ganglia. The scores presented in Figure 11.10 are substantiated by evidence obtained from the radioautographs; this is noted particularly at 6 DIV after ara C treatment. Although the NO scores for controls, PEMF and DC overlap, inspection of the radioautographs find densely-labeled neurons and neurites swirling within the explant prominent in the electrically-stimulated groups. This arrangement of neurites is more dramatic in the NGF-treated ganglia. Further studies to assess numbers and areas of surviving neurons as a function of time in culture and treatment in the presence of ara C, are in progress.

We have preliminary data comparing the numbers of single neurons surviving after 4 DIV in dissociated cultures of DRG; the same degree of stimulation occurred that was found in explant cultures, that is, NGF preserved the maximal number of neurons, while PEMF and DC preserved an intermediate number relative to control cultures. Expanded studies that quantify the numbers of neurons in ara C-treated dissociated cultures are planned. The effect of ara C on neuron survival in dissociated or explant cultures will help to clarify the role that the non-neuronal cells play in maintaining neuronal function. Varon, et al. (1974) have demonstrated that specific proportions of non-neuronal cells are mandatory for neuronal survival in cultures of dissociated DRG. They found that these non-neuronal cells can substitute for NGF.

Application of direct current in vivo with metal electrodes to augment bone repair (cathode in bone) range from 20 µA (in man, Brighton 1979), to 0.5 µA (in rabbits, Spadaro 1979). Levels of 1-100 nA applied by a bimetallic couple (Pt/Ag) to amputated stumps induced partial limb regeneration in adult frogs (Smith 1967) and young rats (Becker and Spadaro 1972; Sisken, et al. 1979). Such experiments with frogs have been confirmed (Borgens, et al. 1977). Roederer, et al. (1983) applied 10 µA DC to transected spinal cord of the lamprey with wick electrodes; cathodally-directed current significantly reduced the normal "die-back" of cut axons. Marsh and Beams (1946) published the first critical study on the effects of applied direct current to nerve regeneration in vitro. They found that current densities of 120 $\mu A/mm^2$ oriented neurite outgrowth to the cathode. More recent studies (Sisken and Smith 1975; Jaffe and Poo 1979; Sisken, et al. 1981; Hinkle, et al. 1981; and Patel and Poo 1982) on chick ganglia, frog neurons, goldfish retina explants, single Xenopus neurons have demonstrated similar orienting effects of the cathode and stimulation of neurite growth. Reported rates of migration of neurons and their processes to the cathode using time lapse cinemicrography are: 100 µm/hr obtained with trigeminal ganglion explants (Sisken and Smith 1975), and 4.8 µm/hr obtained with single Xenopus neurons (Patel and Poo 1982).

In our second series of experiments, sister cultures of dorsal root ganglia exposed to pulsed electromagnetic fields (PEMF) were added. Although many papers exist in the literature on PEMF effects on organ (bone, cartilage models of long bones) and tissue and cell cultures (chondrocytes, osteoblasts, fibroblasts), few laboratories have developed models to test the effects of PEMF on neuronal cell function. Sisken, et al. (1981) reported on differential stimulation of nerve regeneration with a single pulse waveform in comparison to pulse burst signals. Rein, et al. (1982) noted increased noradrenaline release when phaeochromocytoma (PC12) cells were exposed to low frequency electromagnetic fields in vitro. This release was inhibited by 15 mM magnesium thereby implicating calcium ion fluxes in its effects. These results are consistent with those reported by other investigators studying PEMF effects on bone and cartilage cells. Johnson and Rodan (1982) and Korenstein, et al. (1982) have evidence to suggest that PEMF stimulates calcium fluxes and other second messenger activity, similar to that produced by hormone addition.

Using ara C to eliminate the non-neuronal population of cells in the dorsal root ganglia series demonstrates that the sensory neurons possess the inherent ability to regenerate neurites independently of the surrounding cells. This ability is not restricted to sensory neurons; central nervous system

neurons treated with ara C also respond by regenerating their processes (Seil, et al. 1980) as long as they are post-mitotic neurons. Such neurons contain a pool of microtubules necessary for rebuilding the cytoskeleton; new protein synthesis is not mandatory (Yamada and Wessels 1971; Morgan and Seeds 1975; Sisken and Beuerman 1983). The observation that neurite outgrowth begins in ara C medium and continues to lengthen from 3 DIV to 6 DIV in all groups but NGF, suggests that this inherent capability is active for extended lengths of time. The seemingly-paradoxical effects of ara C on NGF neurite outgrowth is clarified by observing radioautographs of such cultures after ^{3}H-proline incorporation. Short, thick processes emerging from the ganglia are heavily-labeled; additionally, layers of labeled processes encircle the centrally-placed neurons. Such a situation was first described by Levi-Montalcini (1966) when excess NGF was present in the culture medium. It is possible that more NGF per neuron is available in the drug-treated cultures since the non-neuronal cells which probably absorb it non-specifically are decreased drastically.

Although neurite production is independently regulated, neuronal survival may ultimately be dependent upon the trophic functions of the non-neuronal cells. Varon, et al. (1974) found that non-neuronal cells can replace the NGF requirement for survival of neurons in dissociated DRG cultures. We have not yet determined neuronal survival in ara C cultures in comparison to those cultures grown in complete medium; it may be that in explant cultures, these accessory cells may also function trophically.

We have shown that application of direct current within limits of 1-20nA stimulates neuronal regeneration of trigeminal ganglia in vitro. Current levels above 20nA are either ineffective or detrimental to the survival of the neurons. By using a mitotic inhibitor, we found that neuronal cells lacking supportive fibroblasts and glia express the inherent ability to regenerate and grow for extended periods of time.

SUMMARY

The effects of various electrical stimuli on nerve regeneration in vitro as defined by neurite outgrowth and neuronal survival, have been documented. A series of experiments were performed on trigeminal ganglia to correlate both parameters of regeneration with different levels of direct current; the current delivered was tested using tantalum or platinum electrodes, or by agar salt bridge electrodes. A dose response curve showed that regeneration was stimulated within a "current window" of 1-20 nA; maximal stimulation occurred with 10 nA. No differences were noted when agar electrodes replaced the metal electrodes, arguing against any involvement of deleterious electrode products. In addition, the growth parameters tested were correlated with current applied rather than voltage imposed. Such trigeminal ganglia stimulated with direct current form "synaptic-type" contacts with the corneal stroma when the ganglia is co-cultured with cornea.

The effects of direct current, pulsed electromagnetic fields or NGF were determined on dorsal root ganglion regeneration as a function of age in culture. In addition, these effects were assessed after treatment with the drug, ara C, to dissociate the electrical or NGF effects on the neuronal cells from those of the non-neuronal cells. Neurite outgrowth was expansive at 3 days in vitro in all groups including the controls; this growth continued to 6 days in vitro demonstrating the inherent ability of neurons to grow in the absence of supportive cells.

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12
Role of Extracellular Matrix Components in Local Control of Bone Regeneration, Remodeling and Repair

A. Hari Reddi

INTRODUCTION

The major aim of most students of tissue repair is to attain predictable and optimal healing of soft and hard tissues. The factors that regulate initiation, promotion and termination of tissue repair are not well understood. A common feature of most wounds is that the healing process is a local phenomenon. Since wound healing is a focal activity it is logical that factors regulating repair processes are locally released at the site of injury. The aim of this article is to demonstrate local induction of bone formation by demineralized extracellular matrix. The growing experimental evidence in support of the concept that extracellular matrix components carry and convey information to responding cells of the microcosm of tissue repair as a cascade will be presented.

CASCADE OF BONE DEVELOPMENT

The endogenous potential of the hard tissues for repair is well known to students of bone biology. The sequence of events during fracture healing in man is common knowledge to most orthopedic surgeons and traumatologists. However, the cell biology of hard tissue repair and the mechanism of action in the initiation of regeneration of bone is not well understood. A biochemical approach to the problem is inherently complicated by the tissue heterogeneity and the technical difficulty of avoiding adjoining bone and connective tissue. However these deterrents may be circumvented by the use of extracellular matrix-induced bone development system (Urist 1965; Reddi and Huggins 1972; Reddi and Anderson 1976; Reddi 1981; Reddi 1983) in an extra-

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Chapter 1

EFFECTS OF ELECTROMAGNETIC FIELDS ON NERVE REGENERATION

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INTRODUCTION

The problem of regeneration in the central and peripheral nervous system has been the topic of numerous investigations and a variety of techniques have been used to effect complete restoration (see Section II. and Spinal Cord Reconstruction, Ed. Kao, Bunge, Reier,1983). In the past 10 years a resurgence of interest has developed in employing electric fields to stimulate regrowth. The purpose of this chapter is to present results obtained with these fields and to explore future avenues of research.

I. IN VITRO STUDIES

The pioneering work of Ross Harrison (1907) demonstrated that isolated nerve cells are capable of forming nerve processes (neurites) when isolated in a dish containing proper nutrient medium (tissue culture). This tissue culture technique has been used to study the effects of various electric fields on neuronal growth. Since the tissues are denervated at the time of culture, the regrowth response is termed "neuronal regeneration" and this regeneration is assessed by determining outgrowth of neurites from the neuronal cell bodies, and survival of neurons with reestablishment of neurotransmitter synthesis and neurophysiological activity.

The first report of electric field effects on neuronal regeneration in vitro was by Ingvar (1920) who found that regenerating cell processes orient along direct current (DC, 0.1-0.2 uA/cm^2) field lines. In 1934 Karssen and Sager

and confirmed these findings using fields less than 1 uA/cm^2. In the same year Paul Weiss, a noted embryologist, maintained that nerves grow along the electrically-induced stress lines of the plasma clot so that growth was only secondarily effected. In 1946 Marsh and Beams presented definitive proof that the neurons were directly influenced by specific levels of galvanic current (120 uA/mm^2). Not only did the newly-formed axons grow preferentially to the cathode, but growth was reoriented in response to cathode placement. Since that time, other studies using direct current have demonstrated stimulation of growth and directional response to the cathode (Sisken and Smith, 1975; Jaffe and Poo, 1979; Hinkle et al., 1981; Freeman et al., 1981, 1985; Patel and Poo, 1982, 1984). More recently, non-invasively induced current via pulsed electromagnetic fields (PEMF) has been applied to cultured neurons resulting in both stimulatory and inhibitory effects (see below).

A. Levels of Applied Current

Two schools of thought appear to exist for applying optimum levels of DC to neuronal systems <u>in vitro</u>. One concept stems from our work with the application of nAmp levels by in-dwelling metal (platinum or tantalum) electrodes for long time periods (Sisken, 1975-85), and the other from the laboratory of Jaffe (Jaffe and Poo, 1979) who applied mAmp levels of DC via agar bridge electrodes for relatively short time periods, the latter technique

introduced by Marsh and Beams (1946). Other investigators, as will be documented, have employed this system. However, it is interesting to note that with either system, cathodally-oriented growth has been observed.

The origin for use of minute fields of 100 uV/cm or less arose from the work of Becker and Murray (1970), and Pilla (1974) who found that application of dc levels of 10^{-8} -10^{-4} V/cm (10 nV- 100 uV/cm) provoked morphological changes in amphibian red blood cells to a "dedifferentiated" state. Pilla observed the greatest degree of change at the anode, the pole wherein calcium ions accumulate. Addition of bulk calcium ions to a solution of RBCs produced the same "dedifferentiated" response in 10% of the cells. Electrochemical tests of different electrodes indicated the superior quality of titanium or tantalum as electrodes; both demonstrated little faradaic current flow over a 2-V potential range. In 1975, Sisken and Smith reported on the stimulation of neuronal regeneration <u>in vitro</u>; minute levels of DC (.001- 11.5 nA/mm^2) applied via point platinum electrodes to trigeminal ganglion cultures enhanced neurite growth that was oriented to the cathode; the rate of growth was 0.1 mm/hr. Survival of these peripheral neurons was also stimulated. These studies have been extended using dorsal root sensory ganglia to include and compare pulsed electromagnetically induced current (PEMF, see below) effects with those produced by applied DC in a non-uniform field (Sisken et al., 1981, 1982, 1984, 1985). Our aims have been to assess electric field effects on neuronal

regeneration including studies on the nerve cell body itself; to this end, we expose our neuronal population to minute levels of DC for extended periods of time (3 days) and observe the effects at this time or after an additional 3 days of culture. The results of these experiments indicate that not only is neurite growth enhanced, but protein content is significantly increased over control cultures (Sisken et al., 1983). These observations are not dependent upon the number of non-neuronal cells (assessed by 3H-thymidine incorporation, unpublished observations). This line of study differs from the studies of other groups who are essentially studying electrophoresis-dependent neurite growth and movement of surface receptors.

More recently we have tested different levels of DC on the peripheral ganglia model using agar or metal (platinum, tantalum) electrodes (Sisken, Barr and Estes, 1984) or tantalum electrodes driven by constant current sources (Sisken and Sisken, 1984). Maximal stimulation of regeneration has been obtained with constant current levels of 10 nA (agar electrodes) or 60 nA (tantalum electrodes) in chick sensory ganglia. Similar experiments using neuroblastoma cell lines (Sisken and Estes, 1984) indicate that greater numbers of cells form processes after application of 10 nA DC; increasing the constant current levels above 10 nA does not increase transformation.

Higher levels of DC, mA) were employed first by Marsh and Beams (as stated above) and then by Jaffe and Poo (1979).

The model developed by Jaffe and Poo used agar electrodes to expose dorsal root ganglia cultured in the presence of nerve growth factor to a uniform electric field created by applying up to 140 mV/mm across the dish for periods up to 20 hrs. Field strengths of 70-140 mV/mm induced faster outgrowth of neurites oriented toward the cathode. The effective average current density in these experiments was 14.3 mA/cm^2 which was in the same range as that used by Marsh and Beams (Table 1). They postulated that the applied electric fields could cause electrophoresis of nerve growth factor receptors to the growing neurite. Hinkle et al., (1981) used a similar system to expose single neurons from Xenopus neural tube. Preferential growth to the cathode was obtained with exposure times of 18-20 hours; threshold values for this response was 6-8 mV/mm. Patel and Poo (1982) applied steady electric fields of 0.1-10 V/cm to single Xenopus neurons and also found stimulated growth of neurites facing the cathode; the number of neurite-containing neurons was increased in these treated cultures as was neurite length. Addition of concanavalin A abolished the electrically-induced effects while fluorescently-labeled Con A receptor accumulated at the cathode. This cathodal accumulation of growth-controlling surface glycoproteins was implicated as the mechanism by which electric fields exert their effect.

The effects of electric fields on central nervous system regeneration in vitro have also been investigated. Khan and Gaik (1984) placed rat embryo spinal cord explants on carbon

fibers (8-10 um diameter) in a culture dish and applied 200 nA DC to the fibers. Cathodally-oriented growth (length and number of neurites) was significantly enhanced in this system. In our laboratory, spinal cord explants of 8 day chick embryos or 16 day rat fetus were treated with 10 nA DC or single pulse 72 Hz PEMF in the presence of varying concentrations of cytosine arabinoside, ara C (Sisken, 1985). The area of the explant, total area of outgrowth and area occupied by neurites was assessed on cultures stained with Bodian silver or on radioautographs of explants showing incorporation of 3H-proline. Both DC (3 day continuously) and PEMF (12 hr/day for 3 day) significantly stimulated neurite outgrowth relative to controls.

Freeman et al., (1985) have described the development and use of their circularly vibrating probe on central (retinal) neurons of the goldfish. This probe is capable of discriminating current densities of 5 nA/cm^2. They provided evidence for currents of 10-100 nA/cm^2 that flow into the filopodia of growth cones of cultured retinal ganglion cells and back out from near the junction of growth cone and filopodia. These currents are produced only during active growth and are believed to be carried primarily by calcium ions. To determine whether such endogenous fields were capable of directing filopodial growth toward or away from a point source of current, retinal ganglion cells were exposed to point sources. Threshold current for orientation was 40 nA (70 mV/cm); endogenous currents generate an axial current

within filopodia of 4 uA/cm^2, with an extracellular electric field of 0.3 mV/cm. These endogenous fields are 2 orders of magnitude lower than those used exogenously to cause lateral electrophoresis of surface macromolecules but the authors suggest they might be involved in polarization and lateral electrophoresis of molecules within the filopodia.

B. Pulsed Electromagnetic Fields (PEMF) Studies

The first studies reporting the effects of pulsed electromagnetic fields on nerve tissue in vitro were those of Bawin et al., (1975). Using 147 MHz, 0.8mW/cm2 weak fields amplitude modulated by slow sinusoidal signals, release of bound $^{45}Ca^{+2}$ was increased maximally at 16 Hz (Bawin et al., 1975). These applied electrical gradients are comparable to intrinsic extracellular electric field potentials of the EEG of 50-100 mV/cm. Continuation of these studies (Bawin et al., 1978) demonstrated that this response was unaffected by changing the calcium concentration of the bathing solution, but was inhibited by addition of H^+. These results indicate that these weak fields affect calcium bound to extracellular negative binding sites and H^+ competes for the same sites. There are many other studies describing PEMF effects on isolated pieces of normal tissue such as those by Wachtel 1979, Wheeler, 1979, Bawin et al., 1984 and Gundersen and Greenbaum, 1985.

Regeneration induced by PEMF applied to goldfish retina cultures for 24 hrs was reported by Schwartz et al., 1983.

PEMF (perpendicular orientation of coils) exposure for 1/2 min at 100 V, 1,000 pps, 5 usec and 100V, 200 pps, 25 usec stimulated exaggerated process regrowth. PEMF-induced regrowth resembled that induced by brain-derived growth factors or glial cell conditioned medium. This study represents the only short (1/2 min.) exposure time found in the literature.

Studies in our own laboratory (Sisken et al., 1984) on the effects of 72 Hz, single pulse PEMF (Electrobiology Inc., N.J.) on chick sensory ganglia indicate that threshold levels of 400 nA/cm2 of induced current significantly enhanced neurite outgrowth. This stimulation was comparable to that obtained with DC application of 9 nA/cm2. Using capacitor plates, Yoshioka et al., (1984) reported that 27 MHz electric fields had no effect on rat sensory neurons at 1, 20, 50 and 610 mV/cm; significant stimulation was observed with fields of 10 mV/cm. Addition of nerve growth factor to cultures treated with 10 mV/cm EF did not change the growth response. In 1985, continuing results from Albert's laboratory indicate that 1 mV/cm electric field repeated at 7 Hz inhibited neurite outgrowth of 14-15 day rat ganglia in the presence or absence of nerve growth factor.

Field effects on non-neuronal cells (Schwann cells) were tested using capactively-coupled electrical fields (60 kHz) at voltages of 3×10^{-4} V/cm to 3×10^{-2} V/cm for 2-4 days (Culp et al., 1984). A myelin marker (gal C) was

preferentially retained at 250 and 375 V; no differences between groups were obtained in ^{3}H-thymidine uptake studies.

The effects of spatially uniform pulsed fields, focally applied DC fields and focally applied pulsed fields on orienting neurite growth from Xenopus neurons was described by Patel and Poo, 1984. Uniform pulsed fields of an equivalent time-averaged field intensity as uniform DC fields produced the same extent of neurite orientation. Monopolar electric current pulses applied focally through a micropipette to neuritic growth cones modulated the rate of neurite growth; negative (sink) current increased growth rates while positive (source) currents were inhibitory. The threshold current density to obtain a growth cone response within 15 min. with focal DC was 0.2-2 pA/um^2; a similar response was obtained with focally-applied pulse current of 4 pA/um^2, 10 Hz.

II. In Vivo Studies

Electrical recordings of transected nerve activity were first reported by DuBois-Reymond (1843). A more detailed study was repeated by Grenell and Burr (1946) using Ag-AgCl electrodes to measure potential differences on the limb surface of rabbits before and after nerve section or in man after ulnar nerve injury. In each case a marked positive shift in potential was obtained, suggesting that such recordings could be used clinically to determine the extent of nerve injury.

A. Effects of Electric Fields on Peripheral Nerve Regeneration

The application of electric fields to injured nerve tissue has been a recent event; a few early studies (Hoffman, 1952; Bodemer, 1964) using DC, or AC, have been followed by a fairly large number of reports. Hoffman (1952) stimulated the spinal cord of large nerve trunks (10-60 min. with 1.5 mA at 50-100 Hz) after transection of the 5th lumbar nerve of rats. Significant acceleration in reinnervation of denervated muscle fibers was found. Bodemer (1964) stimulated nerve fibers of the brachial plexus *in situ* in an attempt to increase nerve activity in an amputated stump; the electrical stimulation resulted in partial regeneration of the limbs in adult frogs. (see S. D. Smith, this volume; also Sisken, et al., 1983 and Borgens, 1977.) A brief report was presented by Romero-Sierra et al. in 1971 on the application of VHF (27 MHz for 5-30 min.) to

non-transected, desheathed sciatic nerves in situ. Histological investigation of such exposed nerves revealed various degrees of demyelination, Schwann cell damage and distortion of collagen fiber pattern close to the nerve. In the same conference, Yorde et al. reported on the ultrastructural effects of DC on cortical synapses in biopsies of the monkey brain. DC (2.5 mA) was applied through surface electrodes for 1-2 min. to the monkey cortex. A biopsy of this cortex was examined and synaptic vesicles counted; the depletion of vesicles after short periods of DC application was correlated with stimulation of synaptic transmission.

In 1976 Wilson used a non-invasive technique to study the effects of pulsed electromagnetic fields (PEMF) on nerve and spinal cord regeneration. He applied an RF signal (Diapulse, 5-120 mW/cm^2) to transected median-ulnar nerves of rats for 15 min./day for 30-60 days. By 30 days, PEMF-treated animals showed significant restoration of nerve conduction activity and the histological presence of large diameter nerve fibers.

The effects of daily stimulation on reinnervation and acceleration of nerve growth after lesioning the sciatic nerve of the rat was reported by Sebille and Bondoux-Jahan, 1980. Using an intensity 10x that to elicit muscle contraction (30 mV at 50 Hz, pulse duration of 1 msec) for 30 min/day via electrodes attached to the leg skin, the toe-spreading reflex was observed and evaluated. Muscle

stimulation significantly increased the rate of recovery. The increased muscle contractions resulting from the applied electric signal probably hastened the functional recovery of the end plate zone when the neurites reach the muscle membrane. A similar paradigm was used by Nix (1982) after crushing the common peroneal nerve of the rabbit. Stimulation of the external digitorum longus muscle with 10-12 Hz for 8 hr/day via implanted electrodes into the muscle increased the time course of contraction and relaxation thus preventing denervation-induced slowing of muscle activity seen in controls.

In 1981, Winter et al. (1981) reported that insertion of Pt/Ag bimetallic electrodes (100 nA) intraluminally stimulated regeneration of transected sciatic nerves. Regeneration was determined by analyzing the compound action potential obtained; only when the cathode was present and distally was the DC effective, Maehlen and Nja (1982) investigated the effects of electrical stimulation of pre- and postsynaptic cells on sprouting after deneravtion in the guinea pig superior cervical ganglion. Preganglionic stimulation on the cervical sympathetic trunk for 1 hr only immediately after denervation (100 pulses at 20 hz every 25 sec) increased the number of axons innervating each ganglion cell. This effect was abolished with hexamethonium thus blocking ganglionic transmission. The findings support a mechanism whereby retrograde trans-synaptic trophic effects are modulated by impulse activity. Part of the stimulus for sprouting after denervation may be enhanced by a brief

period of hyperactivity induced by the electrical stimulation followed by a period of subnormal activity.

In contrast to direct stimulation of nerves via electrodes, Ito and Bassett (1983) subjected the entire body of rats to pulsed electromagnetic fields after transection of the sciatic nerve. The PEMF consisted of Helmholtz aiding coils delivering a repetitive single pulse of 380 usec positive-going, quasirectangular waveform repeating at 72 Hz with an amplitude of 15 mV (ElectroBiology, Inc.). All rats were treated for 12 hours/day. Motor function was evaluated by plantar-flexion force produced by stimulation of the nerve proximally. Return of motor function occurred within 4 weeks after PEMF in contrast to controls at 8 weeks.

Raji and Bowden (1983) tested the effects of PEMF delivered by a Diapulse machine (RF of 10^{-3} W/cm^2) on regeneration of the transected common peroneal nerve in rats. PEMF was administered for 15 minutes daily for periods of 3 days to 8 weeks. PEMF caused significant increases in skin, deep-tissue and rectal temperatures which returned to normal after treatment. The size of the intraneural blood vessels was increased after treatment and the amount of collagenous tissue fibrosis was reduced with PEMF. Most importantly, these studies support those of Wilson (1976) on the PEMF-acceleration of regeneration and maturation of myelinated axons.

Nix and Hope (1983) crushed the motor innervation to

the soleus muscle in rabbits and stimulated the nerve proximal to the lesion with stainless steel electrodes sewn into a cuff placed around the nerve. Stimulation was performed for 4 weeks with a Grass S88 stimulator using rectangular pulses of 0.2 ms duration, frequency of 4 pps. Twitch force, tetanic tension and muscle action potential amplitude measurements were taken pre-and post-operatively for each animal. In each case, significant differences were obtained as a function of treatment and reinnervation was enhanced. The authors concluded that either the nerves grew faster or they established functional connections to the muscle sooner than untreated animals. They implicate the electrical stimulatory effect with maintenance of large myelinated fibers; it is well-known that increased motor activity enhances motor nerve regeneration. The slow frequency pattern used for stimulation was chosen since the soleus muscle is a slow muscle; it could be that fast muscles were reflexly stimulated by this slow pattern.

Preliminary studies were reported on of rats transected sciatic nerves treated with a clinical pulse burst signal (15 Hz, Electrobiology, Inc.) for five days following transection (Parker et al., 1983). Assessment of regeneration indicated a faster return of neurophysiologically-rec rded function and more myelinated axons/mm^2 in the nerve distal to the transection in than untreated control animals.

Singer and Mehler (1983) questioned whether increased 2-deoxyglucose uptake in axotomized motor neurons of the

hypoglossal was associated with increased electrical activity or protein and RNA synthesis. They recorded spike activity in normal and axotomized hypoglossal nuclei and observed 2-deoxyglucose localization radioautographically in the same nuclei. Increased uptake was noted in the axotomized nucleus but no differences were observed in numbers of action potentials. The authors conclude that increased uptake associated with axotomy was not the result of increased action potential activity but rather correlated with synthesis of protein, RNA and lipid during regeneration.

The effects of PEMF on regeneration of the common peroneal nerve of the cat was determined using a multidisciplinary approach (Orgel et al., 1984). Five days after transection, the cats were exposed to PEMF for 10 hours/day, 6 days/week for 12 weeks. Two different signals were tested: a pulse burst signal used clinically for bone repair (15 Hz, 380 us positive, 24 us negative) and a single repetitive pulse 200 us positive, 6 ms negative, 72 Hz). Electrophysiologic data was collected pre-and post-operatively. Muscle biopsies were taken for fiber typing, the nerves biopsied for fiber counts, and retrograde transport of horseradish peroxidase to the motorneurons in the spinal cord were used for assessing regenerative events. No significant differences were noted between controls and either PEMF signal in: muscle fiber diameter, numbers of fibers/mm2, axon fiber caliber, areas of nerve compound

action potential or muscle compound action potential. However, the numbers of motor neurons retrogradely-labeled in spinal cords of cats treated with the pulse burst signal (15 Hz) were significantly increased (96.8% of the unoperated side). This study represents the most in-depth exploration of electrophysiological and morphological/ morphometric parameters on peripheral nerve regeneration.

Pomeranz et al., 1984 reported on accelerated sprouting of intact saphenous nerves after sciatic nerve transection. Electrical fields were applied by 1 uA DC or AC (20 Hz, 1000 uA/pulse) delivered through stainless steel electrodes placed in the skin of the digit of the hindpaw. Only distally-placed cathode electrodes were effective; no description of how the electrical signals were generated was given. Roman et al. (1985) applied 10 uA DC to rat sciatic nerves transected and containing a 5 mm gap. Proximal and distal ends of the nerve were placed inside a Silastic tube; the cathode-stimulator (Pt wire) was inserted into the tube close to the distal stump. Constant current (1 uA/cm^2) was administered by a battery and variable resistor. The contents of the Silastic tube were fixed at 3 weeks and examined histologically. A good portion of the DC-treated tubes were filled with blood vessels as well as axon bundles; the total contents were 2x larger than controls. The number of myelinated axons per tube was increased by a factor of 3. In another series the tube contained the proximal stump and the cathode stimulator only; no distal stump was present. The 10 uA current alone stimulated

growth of nerve bundles.

B. Effects of Electric Fields on Spinal Cord Regeneration

In 1976 Wilson provided preliminary evidence on the effects of PEMF via Diapulse signals (see above) on hemicordectomies of the upper lumbar segment in cats. The experimental protocol was to expose the cats to diapulse therapy for 30 min/day for 30 days using 50 mW/cm2, 400 pps. Three months after lesioning, the cords were fixed and sectioned for histology. PEMF-treated cords exhibited decreased scarring and regenerating neurites traversing the lesioned area.

Cohen and collaborators (Roederer et al., 1983) investigated the effects of applied DC (10 uA) on rats of regeneration of lamprey spinal cord neurons. Wick electrodes were placed distal to the lesion and current delivered for 5 days. Axonal die-back was significantly correlated with the direction of the applied current; die-back of axons was increased with the anode and decreased (promotion of regeneration) with the cathode. The authors propose (die-back) to be associated with entry of cations (primarily calcium ions) into the transected stump; applied DC interacted with endogenous currents so that increasing (regeneration) or decreasing cation flow resulted in enhancement (degeneration) or reduction of axonal die-back.

A contusion-injured cat model has been used extensively by W. Young in his basic studies on spinal cord

injury. Recent application of PEMF (Gruner and Young, 1985) with the Diapulse signal (400/sec 65 usec pulses of RF 27.12 MHz, average power of 100mW/cm2) on this model indicates that cats treated for 1 hour daily with PEMF 4 hrs. post-injury (but not 1 hr) demonstrated beneficial effect on preservation and maintenance of function. Short term analyses of PEMF on ionic changes when administered 3 hrs post-injury for 2 hrs had no effect on sodium, potassium or water content of the contused area while it significantly decreased calcium accumulation in the cord (Young, 1984; Young et al., 1985). These results support the beneficial effects of PEMF on restoration of peripheral nerve function cited above and provide another example of electric field action on decreasing calcium entry into injured spinal cord.

Functional electrostimulation (FES) has come of age following the original design of the pacemaker. Different types of FES are now in use or in different stages of development for treating various clinical states. FES has been used to: alleviate pain, induce artificial respiration (diaphragm pacing), cause contraction (continence restoration) or evacuation (micturation reflex) of the bladder, and inhibit muscle atrophy and improve motor function.

III. THEORIES OF MECHANISM OF ACTION OF ELECTRIC FIELDS

The following discussion on mechanisms of action of imposed electric fields strongly implicates the role of calcium ions, and models originally proposed by Pilla (1974), Jaffe et al., (1974) and Bawin et al., (1975) have addressed this point. In 1974, Pilla proposed a theoretical model to explain electrical effects on tissues and cells indicating that nonneutral charges exist at the interface of the cell membrane and intra- and extracellular fluids. Highly charged electric fields exist at this site and energy levels of 10^{-3} W are capable of perturbing this interfacial structure. He suggested that alterations of a few millivolts change could result in gross alterations of specifically adsorbed or bound species resulting in electrochemical information transfer. He demonstrated that current levels of 10 nV/cm - 100 uV/cm) of DC produced morphological changes in RBC (at the anode) and implicated the slowly-migrating calcium ions in this phenomena. This hypothesis was reinforced when addition of calcium alone to these cells induced similar morphological changes. At the same conference, Jaffe et al., (1974) proposed an electrical hypothesis for localized growth suggesting that the plasma membrane of a growth region becomes relatively leaky to cations such as calcium, magnesium, sodium and hydrogen which exist at higher concentrations extracellularly than intracellularly. As the cations enter the growth point of the cell, an electric current/field is generated. Such a

field would generate movement pulling more negative cytoplasmic substances and vesicles (which form new membrane) toward the leaky area, thus generating more leaky membrane. This "positive feed-back loop" would increase the probability of localized growth and membrane expansions. This hypothesis was tested by measuring electric currents around developing fucoid eggs, finding growth points of current entry which contain calcium and sodium components. Application of fields of 200 mV/egg to naive eggs induced blister "fertilization-like" vesicle formation at the positive side of the egg.

Bawin et al., (1975) implicated a specific class of calcium sites at the extracellular neuronal membrane responsive to low frequency electric fields and postulated to play a role in regulating cell excitability. They found that RF fields modulated at specific brain wave frequencies increased calcium efflux from calcium^{45}- loaded cerebral cortex and that efflux was enhanced in the presence of additional H+ ions. Since that time, other interpretations of bioelectric effects of external electric fields have been reported and are reviewed in the following section.

A. Levels of DC 100 uV/cm and below

As a consequence of the work of Becker and Murray (1970) and Pilla (1974) who described morphological changes in red blood cells using nA levels of current, we began our studies on assessing the effects of these minute fields on cultured neurons (Sisken and Smith, 1975). The stimulation

of neuritic growth which was cathodally-oriented by these fields was postulated to be correlated with changes of calcium ions at the boundary of the cell membrane. Studies on the growth cones of cultures subjected to DC (Sisken and Lafferty, 1978) indicated that they were also enlarged. Calcium flux changes as a result of these fields (Sisken et al., 1981) indicated that application of DC (~10 nA/cm^2) to trigeminal neurons significantly increased calcium efflux of preloaded ganglia relative to control or nerve growth factor-treated ganglia. Our hypothesis was that the DC acted by decreasing calcium entry into cells by enhancing calcuim binding to the external membranous pool.

We have tested the influence of calcium ions on growth processes by blocking calcium entry with lanthanum chloride or the drug Verapramil, or increasing calcium entry with added calcium chloride or the drug A23187 which opens calcium channels allowing intracellular calcium concentration to increase. These studies indicate that long-term application (6 days) of compounds which inhibit calcium influx (lanthanum, Verapramil) increase neurite formation while those which stimulate calcium influx (added calcium or the ionophore A23187) inhibit neurite formation (Sisken and Sisken, 1984). These results are consistent with those of Bray et al., (1978) who found that low extracellular calcium induced the formation of growth cones all along the length of cultured sensory neurons. It appears very probable that long exposures to minute levels

of DC mimic the drug-induced reduction of calcium entry into the neurons; both result in stimulation of nerve process growth.

The role of cations in neuronal growth and differentiation is slowly becoming clear. The regulation of sodium and potassium levels and Na^+,K^+-pump activity by nerve growth factor (NGF) in early stages of neuronal regeneration in culture has been characterized (Skaper and Varon, 1981). Micromolar levels of calcium is required for growth cone formation (the growth end of neurites) and mobility (actin network), but is not required for neurite formation and (microtubule) stability.

An increase in the number of calcium channels has been correlated with active neurite outgrowth as neurons differentiate (Fukada and Kameyama, 1979; Freeman et al., 1985). This calcium requirement is reduced as a function of time in culture. Although the precise role of calcium in neurite formation is not known, Hammerschlag (1977) postulated that amino acid uptake and protein transport are dependent upon calcium entry through the cell membrane, and Fukada and Kameyama (1979) suggested that increased calcium is needed for protein syntheis for neurite membranous growth. In an elegent study, Anglister et al., (1982) detected calcium action potentials in neuroblastoma cells and found that voltage-activated calcium channels are less abundant in neurite processes, and more abundant in growth cones. Upon depolarization with excess potassium or electrical stimulation, the area of growth cones increased

by 20-120% associated with increased neurite outgrowth. This expansion in growth cone area was inhibited with cadmium (calcium blocker) or in low-calcium media. The authors suggested that calcium ion entry functions as a trigger for neurite elongation.

Freeman (1985) has employed a novel circularly vibrating probe (capable of measuring current densities of 5 nA/cm) to measure the direction and magnitude of endogenous current at the growth cone. Steady or slowly varying (not pulsatile) currents in the range of 10-100 nA/cm^2 enter the filopodia tip of the growth cone at the end of a neurite and flow down it and back out toward the base. The current appears to be carried primarily by calcium ions and is not derived from growth cone motion or flow of medium over the surface of the plasma membrane. Average strengths of 70 mV/cm of applied fields were necessary to orient the growth cones to the cathode; the magnitude of the applied fields is 2 orders larger than those found endogenously. The authors conclude that the endogenous currents are too small to cause lateral electrophoresis of surface molecules but may be the result of localization of clusters of voltage-sensitive calcium channels at the tips of growth cones. These calcium channel proteins are brought to the growing nerve tips by active transport and are incorporated into newly formed, expanding membrane; sodium channels arrive later due to a "slower rate of lateral diffusion in the plasmalemma". Expanded growth of the plasma membrane that occurs by fusion

of intracytoplasmic vesicles to the membrane requires calcium, some of which is current-generated. Other functions assigned to calcium ions include alignment of actin and myosin in the growth cone and subsequent calcium-dependent contractile movements. Small changes in the membrane potential resulting from external electric field application affect calcium ion entry. Depolarization on the side of the cell facing the cathode would increase calcium entry, while that facing the anode would inhibit entry. Excess calcium entering the growth cone not used for membrane expansion would be extruded as exocytosis of intracytoplasmic vesicles occurs, thereby maintaining calcium homeostasis.

The paradigm presented above by Freeman et al., is most provocative; the sequence of processes suggested to occur at the level of the neuronal growth cone could be applied to other tissues and cells and should be considered by other workers in this area. In this context, a hypothesis to explain stimulation of regeneration as a function of minute levels of direct current (~10 nA) as studied in our own laboratory incorporate some of this thinking. The fields applied to neuronal (sensory/motor) explants are so small that membrane depolarization is perhaps in the order of ~1mV or less; attempts to measure small changes has yielded equivocal results (Sisken and Ringham, unpublished results). However, the levels of DC used in our system (~10 nA/cm^2) is equivalent to that measured by Freeman et al., 1982 (10-100 nA/cm^2) flowing into the tips of growth cones of retinal neurons. Adding any additional current to that present

endogenously would increase calcium entry in growth cones facing the cathode; the cascade of events postulated by Jaffe (1974) and Freeman et al. (1985) would ensue, resulting in growth cone expansion. Such expansions with microspikes have been observed (Sisken and Lafferty, 1978) and are demonstrated in Figure 1. Orientation of the newly-formed growth cones to the cathodes by motile (calcium-dependent actomyosin system) growth cones would continue with ensuing lengthening of the neurites; such activity is normally suppressed as the neurons mature (Cooper and Schliwa, 1985). Calcium entry would also be instrumental in stimulating amino acid uptake and protein synthesis (as observed,Sisken and Lafferty, 1978; Sisken and Estes, 1983) needed for membrane proteins and microtubule formation. Exposure to direct current over hours would be manifest by increased neurite outgrowth. As the neurons differentiate in culture, calcium channels are replaced by sodium channels (Fukada and Kamyama, 1979; Anglister et al., 1982) thereby reducing calcium entry . Continued application of minute levels of DC may act to increase the adhesion of non-entering calcium to the external membrane (Sisken et al., 1981). Reduction of excess calcium entry also serves to stabilize the microtubules in the neurites. The foregoing activities require that the parent neuronal cell body be synthesizing relatively large amounts of protein to enable the processes to form and elongate; neurites of lengths of 100-150 um have been observed in our DC-treated cultures. We

have found that the protein content in such explants are significantly larger than control explants and approach levels of NGF-treated sister cultures (unpublished observations).

The paradoxical role of DC may be related to the presence of calcium channels (Freeman, et al., 1985; Cooper & Schliwa, 1985); when present, DC increases calcium intracellularly by adding to preexisting endogenous (calcium) currents. When sodium channels replace calcium channels, calcium entry is regulated precisely by transport processes and depolarization phenomena. Addition of nA levels of DC to relatively more mature neurons does not increase calcium entry, rather it fosters binding of the calcium to the negative sites on the external membrane.

B. Levels of DC 10 mV/cm to 1.5 V/cm

The agar wedge system employed by Marsh and Beams (1946) was modified by Jaffe and Poo (1979) to apply field strengths of ~100 mV/mm. This model has been used by a number of investigators (Section I) who have examined the role of field strengths up to 1.5 V/cm on neurite growth and orientation. Jaffe and Poo (1979) suggested that such fields caused lateral electrophoresis of nerve growth factor receptors along the plasma membrane. Hinkle et al. (1981), however, favored the hypothesis that the imposed electric fields acted on the interior of the growth cone since the voltage drop across the microspikes of the growth cone was of the same magnitude (~0.7 mV/cell) as that

found to oreint neurites. The mechanism of action of these fields on neurite growth orientation most likely does not involve field-induced mechanical effects or chemical gradients (Patel and Poo, 1982) since constant perfusion with fresh medium did not change the observed effects. They proposed three mechanisms to account for oriented neurite growth; (1)an electric field-induced potential change causing redistribution of cytoplasmic material, (2)alteration of membrane potential asymmetry inducing preferential growth, and (3) an electrophoretic redistribution of charged surface molecules in the plasma membrane. Data obtained from their experiments favored the third hypothesis since field strengths used for neurite growth caused fluorescently-bound Con A receptors to accumulate on the cathodal side of the neuron, and inhibition of receptor migration abolished the orientation effect.

The electromigration model (Fraser and Poo, 1982) proposes receptor (acetylcholine, ACh) migration to the synaptic region. Transient fields of 1 V/cm are generated normally by synaptic currents; such field strengths applied exogenously cause electrophoretic migration of ACh receptors to synaptic contact areas. The proposal offered by these investigators states that "transient electric fields associated with neuronal activities serve to develop, maintain and modulate the topography of the membrane components responsible for these activities".

Finally, the role played by calcium ions in altering

locomotory activity after imposition of 0.5 - 15 V/cm electric fields action was addressed by Cooper and Schliwa (1985). Migration of keratocytes to the cathode was observed under these fields, and inhibited with calcium channel antagonists. Their hypothesis for cathodal migration requires asymmetric calcium entry into the cell through specific calcium channels to trigger contractile activity and direct orientated cell movement. In addition, they discuss the contradictory role played by calcium, specifically in neuronal systems. Anodal neurite retraction (observed by Patel and Poo, 1982) in field strengths of 5 V/cm hyperpolarized the neuron resulting in calcium entry and its subsequent axoplasmic filament degradation. However, depolarization at the cathode also increased calcium entry, with consequent increased growth cone formation and neuronal survival. They did not resolve this discrepancy; it may finally rest on the absolute concentration of intracellular calcium levels which is as dependent upon calcium extrusion as it is on calcium entry.

C. Pulsed Fields

The action of various pulsed fields on nerve tissue has been explored and the association of these effects with calcium changes has been implicated. Efflux studies of calcium after application of low frequency extracellular fields (ELF = 1-300 Hz, Bawin et al., 1976, 1978; Blackman et al., 1982) demonstrated a correlation of enhanced efflux with specific intensity ranges. The amplitude range and

frequency of these fields are comparable to those of extracellular brain waves. The hypothesis offered by Bawin et al., 1978, proposed that specific negative sites on the plasma membrane are occupied by calcium, and are affected by applied ELF.

Comparative studies of DC and PEMF on promotion of neurite growth (Sisken et al., 1984) correlated stimulatory effects with current density (Ø.7 uA/cm^2) and orientation of the Helmholtz coils; only coils oriented so that the magnetic field was parallel to the bottom of the dish (PEMF-V) produced a significant response. Effective levels of DC and PEMF-V (per polarity) were equivalent ($1Ø^{-3}$ coulombs) on a time exposure basis. Mechanisms associated with the PEMF fields may also involve calcium ion changes (as discussed previously for DC effects) since the effective dosage of both systems are in the same range.

Threshold levels of 3-3Ø mV/cm DC or 4 pA/um^2 pulsed fields applied focally to influence the direction of neurite growth were found to be similar to those associated with action potentials, and synaptic activity (Patel and Poo, 1984). They suggest that the action of these fields is not to generally guide nerve growth in developing systems, but to modulate localized neurite orientation to specific areas of intense activity. In another study, square wave pulses comparable to local extracellular AC fields produced by nerve action potentials (1ØØ Hz, Lin-Liu et al., 1984) redistributed Con A receptors to the cathodal area of cultured myoblasts. These findings add support to the

electromigration model proposed by Fraser and Poo, 1982 that electrical events associated with nerve activity can modulate neuronal topography.

IV. FUTURE STUDIES

In view of the rapid technological advances and the increasing numbers of workers in the area of bioeffects of externally-applied electrical fields, it is anticipated that more clinically-useful procedures will be developed to address nerve regeneration problems. Hopefully these methods will encompass stimulation of regeneration in the spinal cord as well.

Basic studies to determine the mechanism of action of these fields and how such imposed fields relate to normal activity and to activity following degeneration and regeneration should be conducted in parallel with the clinical investigations. It is hoped that advances made in the area of soft tissue regeneration in general, and nerve tissue specifically, will duplicate or exceed that found in the electrical treatment of bone repair.

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Table 1

Values of Electric Field (E),

Current Density (J) and Time (Hrs)

for *in vitro* Experiments on Neuronal Tissue

	E	J	Hrs
Ingvar (1920)	?	.0015 uA/mm^2	?
Marsh and Beams (1946)	65 mV/cm	12 mA/cm^2	29
Sisken and Smith (1975)	80 uV/cm	11.5 nA/mm^2	96
Sisken (1984)	0.6 uV/cm	9 nA/cm^2	72
Jaffe and Poo (1979)	1000 mV/cm	14.3 mA/cm^2	4-8 (20 max)
Hinkle et al. (1981)	70-1900 mV/cm	1-27mA/cm^2	18-20 hrs
Patel and Poo (1982)	100-10,000 mV/cm	1.4-143 mA/cm^2	6
Patel and Poo (focal) (1984)	3-30 mV/cm	0.2-2 pA/um^2	.25
Freeman (1985)	70-350 mV/cm	~1 mA/cm^2	

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FIGURE LEGEND

Scanning electron microscopy of neurite growth cones of 8 day chick embryo trigeminal ganglia after 4 days *in vitro*. The growth cones (gc) were found at the edge of the explant lying on top of the fibroblast mat. Note the expanded growth cones of the DC-treated ganglia.

A (control culture) and B (10 nA DC-treated culture) X2400

C (control culture) and D (10 nA DC-treated culture) X9750

Table 1

Values of Electric Field (E),

Current Density (J) and Time (Hrs)

for *in vitro* Experiments on Neuronal Tissue

	E	J	Hrs
Ingvar (1920)	?	.0015 uA/mm^2	?
Marsh and Beams (1946)	65 mV/cm	12 mA/cm^2	29
Sisken and Smith (1975)	80 uV/cm	11.5 nA/mm^2	96
Sisken (1984)	0.6 uV/cm	9 nA/cm^2	72
Jaffe and Poo (1979)	1000 mV/cm	14.3 mA/cm^2	4-8 (20 max)
Hinkle et al. (1981)	70-1900 mV/cm	1-27mA/cm^2	18-20 hrs
Patel and Poo (1982)	100-10,000 mV/cm	1.4-143 mA/cm^2	6
Patel and Poo (focal) (1984)	3-30 mV/cm	0.2-2 pA/um^2	.25
Freeman (1985)	70-350 mV/cm	~1 mA/cm^2	

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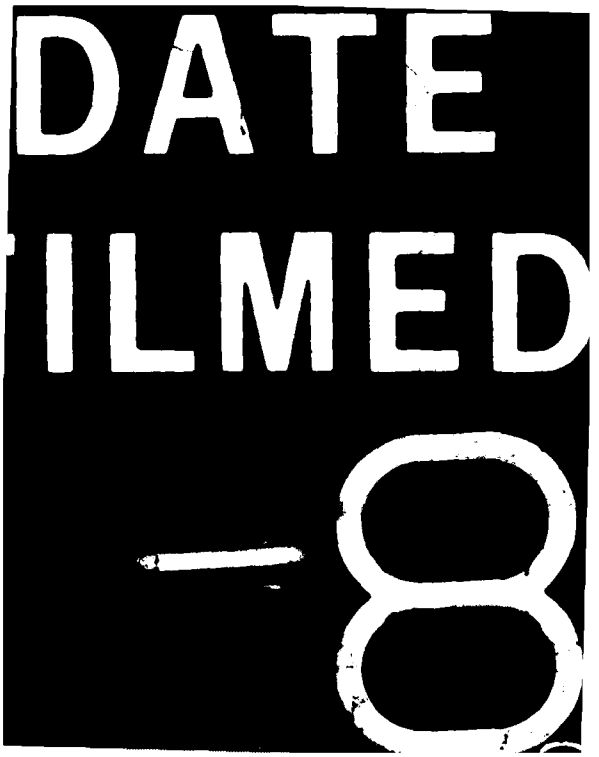
c
m
d
g
p
m
q
g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